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Investigating the Integration-Independent Role of HIV-1 IN in the Viral Life Cycle

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by

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ABSTRACT OF THE DISSERTATION

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Professor Sebla B. Kutluay, Chair

Human immunodeficiency virus type 1 (HIV-1) relies on a handful of essential enzymes for replication. Among these, the viral integrase enzyme (IN) plays a pivotal role in the viral life cycle by catalyzing the integration of the reverse-transcribed viral DNA into the host chromosome. While integration is the canonical role of IN, new research has uncovered an additional vital role for IN during virion morphogenesis. This dissertation elucidates how IN contributes to proper packaging of the viral RNA genome (vRNA) within the viral capsid and examines the fate of improperly formed viral particles in target cells.

IN is proposed to mediate proper placement of the vRNA within the capsid in mature virions by binding to vRNA at a defined binding site in its C-terminal domain. Mutations at the CTD RNA-binding site lead to the generation of morphologically aberrant virions with vRNA mislocalized outside of the empty capsid lattice, suggesting that IN-RNA binding is necessary for proper virion maturation. However, multiple IN mutations outside of the RNA-binding site cause the same morphological defects in virions, suggesting that another property of IN, such as its multimerization, may be responsible for its role in virion morphogenesis. In Chapter 2 we dissect the contribution of IN-RNA binding and IN multimerization to virion morphogenesis and demonstrate that IN-RNA binding accounts for the role of IN in virion maturation, although IN tetramerization is likely a prerequisite for RNA binding to occur. We further identify three separate mechanisms by which IN-RNA interaction can be inhibited, all of which prevent

proper virion maturation. In Chapter 3, we provide evidence that the morphological defects caused by IN mutations lead to the premature loss of the exposed vRNA and IN itself in infected target cells, preventing further viral replication.

As a whole, this dissertation provides mechanistic insight into how IN contributes to virion morphogenesis by establishing IN-RNA binding as the determining factor by which IN ensures proper placement of the vRNA in viral particles, while highlighting the importance of proper IN multimerization for RNA-binding. This work also provides an explanation for the common block in viral replication observed in many IN mutant viruses.

Chapter 1:

Introduction

1.1 Introduction

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of AIDS, and since its discovery in 1983[1,2] has become one of the leading causes of the death worldwide due to infectious disease. Intensive study of the HIV-1 life cycle has led to the identification of a handful of viral enzymes required for virus replication, and antiretroviral compounds that specifically inhibit the functions of these enzymes have transformed HIV-1 infection from a death sentence into a manageable disease. The HIV-1 integrase enzyme (IN) plays a vital role in the viral life cycle by catalyzing the integration of viral DNA into the host chromosome. This function has been successfully targeted by a class of antiretrovirals known as integrase strand-transfer inhibitors (INSTIs)[3,4]. Four FDA-approved INSTIs –raltegravir[5], elvitegravir[6], dolutegravir[7], and bictegravir[8]- have become key components of anti-retroviral therapy regimens and are both highly effective and well tolerated[4,9-11]. A fifth –cabotegravir[12]- is currently in late stage clinical trials. However, despite high barriers with the second-generation INSTIs, treatment does select for drug resistance[13-15] and mutations conferring resistance to multiple INSTIs have been reported in clinical settings[16,17], highlighting the need for continued research and development of both improved and novel antiretroviral compounds.

It was recently discovered that IN has a second essential role in the HIV-1 life cycle during virion morphogenesis. IN binds the viral RNA (vRNA) genome in virions and is necessary for the proper placement of vRNA within the viral capsid lattice during virion maturation[18]. Loss of IN-RNA binding leads to mislocalization of the viral genome in virions and prevents viral replication in target cells[18]. This discovery opens up new avenues for therapeutic targeting of the second function of IN that is independent of its already targeted catalytic function. Here, I briefly review the HIV-1 life cycle, and then detail the roles IN plays in both integration and virion morphogenesis. A better mechanistic understanding of how IN functions in the HIV-1 life cycle can help inform the development of improved and novel antiretroviral drugs.

1.2 Overview of the HIV-1 life cycle

The HIV-1 life cycle can be broadly divided into an early stage (up to integration) and a late stage (after integration). Mature HIV-1 virions consist of two copies of single-stranded RNA genome and replicative enzymes (reverse transcriptase (RT) and IN) encased in a conical protein lattice made up by the viral capsid (CA) protein, together forming the viral core. The viral genome inside the core exists in the form of a viral ribonucleoprotein complex (vRNP) bound and condensed by the viral nucleocapsid (NC) protein, and associated with RT and IN enzymes[19]. The viral core itself is enclosed within the viral lipid envelope derived from host cell plasma membrane. During budding the virion acquires the envelope (Env) glycoprotein trimers at its surface, which serve as receptors to mediate viral entry[20]. During entry Env engages the CD4 receptor and CXCR4/CCR5 coreceptors on the surface of the target cell, which are present predominantly on CD4+ T cells and cells of the monocyte/macrophage lineage[21,22]. Receptor binding induces a series of conformational changes in Env, resulting in membrane fusion and release of the viral core into the cytoplasm[21,22].

After entry, the viral core is transported towards the nucleus along microtubules[23,24] and reverse transcription ensues. The viral reverse transcriptase enzyme (RT) binds the single-stranded viral RNA and uses it as a template to produce the linear double-stranded viral DNA (vDNA)[25,26]. During this stage, the core undergoes an uncoating process in which the capsid dissembles and CA monomers are shed from the lattice[27]. While uncoating and reverse transcription have long been thought to occur in the cytoplasm, recent studies have provided evidence that these processes are not completed until after nuclear entry[28,29]. IN remains associated with the reverse transcription complex and following completion of vDNA synthesis, a multimer of IN binds to both ends of the linear viral DNA to form the intasome, or the stable synaptic complex. The intasome complex is then transported into the nucleus, where IN catalyzes the integration of the viral DNA into the host cell chromosome[30].

After the viral DNA is integrated into the host chromosome it serves as a template from which single full-length viral mRNAs are transcribed by the host RNA Polymerase II machinery[31]. This viral transcript can remain unspliced or undergo a complex series of splicing events, creating over 50 splice

variants of two size classes (1.8 kb and 4 kb)[31]. The fully spliced 1.8 kb class of HIV-1 mRNAs code for the regulatory Tat, Rev, and Nef proteins and are exported from the nucleus via the NXF1/NXT1 pathway [32-34]. The partially spliced 4 kb class of mRNAs code for the viral envelope Env and accessory proteins Vif, Vpr, and Vpu, while the unspliced full-length HIV-1 mRNAs can be packaged into virions as the genomic RNA or translated to generate the major structural proteins, Gag, and the Gag-Pol polyprotein[32-34]. Translation of Gag-Pol, which additionally codes for the replicative enzymes protease (PR), RT, and IN, depends on programmed ribosomal frameshifting of the Gag ORF [35,36]. Both partially spliced and unspliced HIV-1 RNAs are retained in the nucleus until they can be exported in a Rev-dependent fashion[37,38]. Once translated in the cytoplasm, the Rev protein is shuttled back into the nucleus where it binds the Rev response element (RRE) present in HIV-1 partially spliced and unspliced transcripts, and exports them from the nucleus through a CRM1-dependent pathway[39-41].

Unspliced dimeric vRNA is trafficked to the plasma membrane by Gag and this complex subsequently nucleates the assembly of nascent virions[42-44]. During this process, the Gag and Gag-Pol polyproteins polymerize around the vRNA, acquire Env glycoproteins recruited to the budding site and virions bud off from the infected cell in an immature state. During or shortly after budding the virion undergoes a maturation process, in which the Gag and Gag-Pol polyproteins are cleaved into separate mature proteins by the virally encoded PR enzyme. This triggers a structural rearrangement within the virion, whereby the cleaved NC proteins condense the vRNA together with RT and IN to form the viral ribonucleoprotein complex (vRNP), the viral CA lattice assembles around the vRNP, and the now mature virion is ready to infect a new target cell and reinitiate the viral life cycle (**Fig. 1**)[42,43].

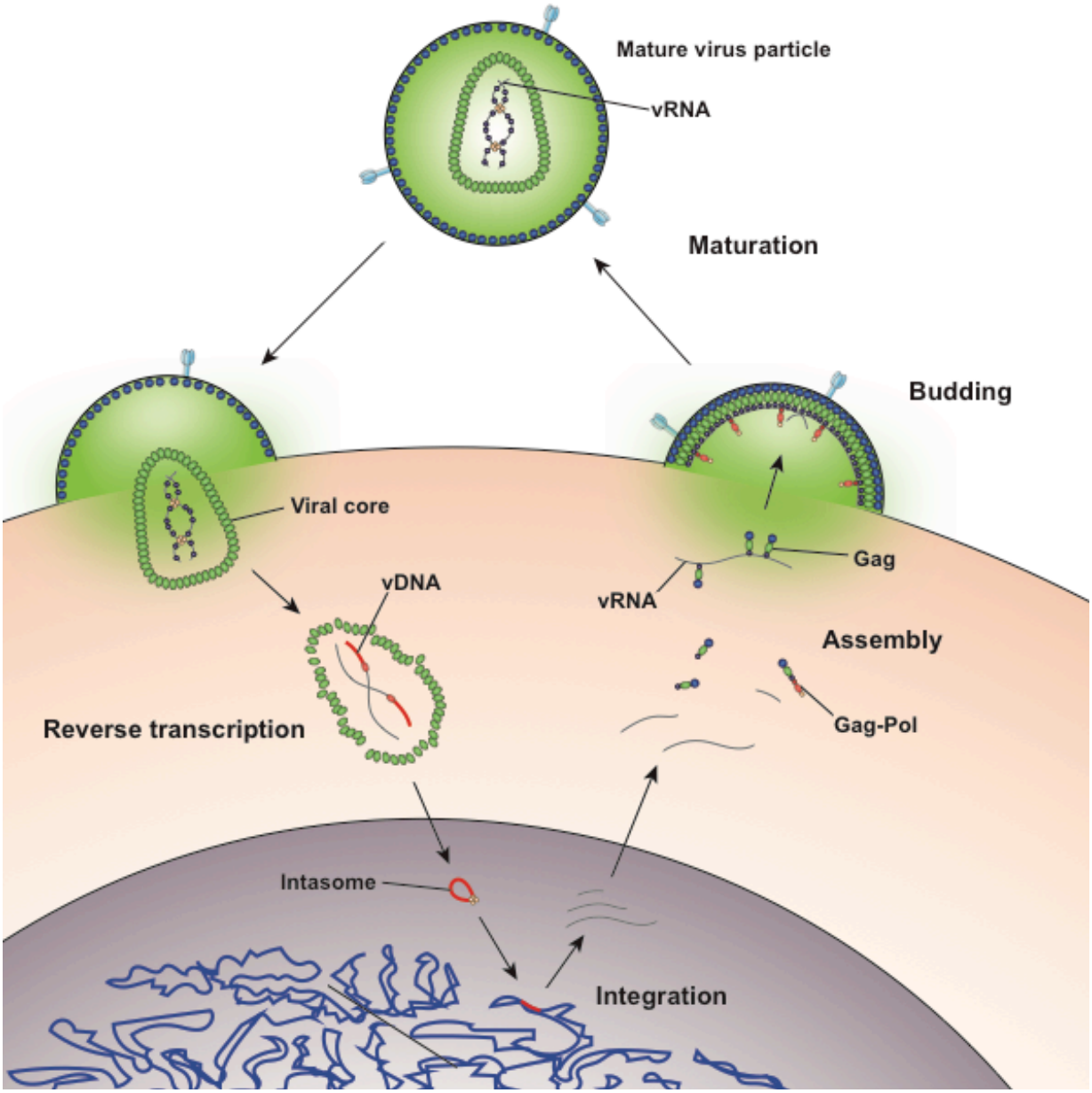

Figure 1: Overview of the HIV-1 life cycle
HIV-1 virions contain two copies of a single-stranded RNA (vRNA, in gray) genome enclosed inside a conical capsid lattice or core. After viral entry the viral core is transported towards the nucleus and the vRNA is reversed transcribed into double-stranded DNA (vDNA, in red.) In the nucleus the vDNA is integrated into the host DNA and serves a template from which more vRNA is transcribed. vRNA and viral protein assemble at the plasma membrane and bud of from the cell as immature virions. During virion maturation the capsid lattice reforms again around the viral genome.

1.3 Integrase in integration

A defining feature of the retroviral life cycle is integration of the reverse-transcribed viral DNA into the host chromosome. During integration, a multimer of IN binds either end of the linear viral DNA to form the intasome complex, which inserts the reverse-transcribed vDNA into the host DNA[30,45]. This function of IN was identified shortly after the discovery of HIV-1 in the early 1980s[46-49], and has been extensively studied. Each IN molecule is composed of three functionally distinct domains: a N-terminal domain (NTD), a catalytic core domain (CCD), and a C-terminal domain (CTD). The NTD and CTD domains mediate DNA binding and play important structural roles in the intasome complex, while the CCD contains a highly conserved D,D,-35-E motif in the enzyme active site necessary for catalytic activity[30,45]. Mutations at these conserved residues, collectively referred to as class I IN mutations[50-52], predictably abolish the catalytic activity of IN in vitro[50] and block the viral life cycle at the integration stage in infected cells[52].

IN is a dynamic protein, and can form a population of monomers, dimers, and tetramers *in vitro*, and as noted above forms multimers during integration[53]. Early studies indicated that IN may function as a multimer by demonstrating that catalytically inactive mutant IN proteins bearing substitutions at different sites could trans-complement each other and regain catalytic activity i*n vitro*[54-56]. More recently, a mechanistic study using a small molecule inhibitor found that the compound binds at the interface between two dimers within an IN tetramer, and interferes with the exchange of IN subunits in a manner that correlates with its ability to inhibit IN catalytic activity, providing further evidence that proper IN multimerization is critical for its function[57].

The first retroviral intasome to be structurally characterized was that of the prototype foamy virus (PFV) from the spumavirus genus, which consists of a tetramer of IN made up of a dimer of dimers with viral DNA between the two subunits (**Fig. 2**)[58-60]. Each dimer includes an inner and outer IN molecule, with the inner subunits interacting with the viral and host DNA. The catalytic site in the inner IN CCD cooperatively coordinates the integration reaction with the NTD of the opposing inner IN, while the inner

CTDs bind the host DNA and help hold the two dimers together. Meanwhile, the outer IN subunits further stabilize the complex by contacting the inner IN molecules at the CCD-CCD interface.

It was generally assumed that the HIV-1 intasome complex shared a similar structure. Tetrameric IN binds to viral DNA in cross-linking experiments[61], is catalytically active *in vitro*[62], and has been observed to interact with viral DNA by atomic force microscopy[63,64]. However, recent studies have suggested that HIV-1 IN forms even higher-order multimers within the intasome complex[65]. While detailed structural analysis of the HIV-1 intasome has long been hindered by the propensity of HIV-1 IN to aggregate in solution, the aforementioned study overcame this issue by generating a hyper-active HIV-1 IN mutant protein with improved solubility. Single particle cryo-EM structures of the HIV-1 IN construct in complex with DNA indicate that while the basic architecture of the IN tetramer is conserved across PFV and HIV-1, in HIV-1 a higher order multimer of several tetramers may be needed to efficiently integrate viral DNA, although a lower-order intasome consisting of an IN tetramer was also observed (**Fig. 2**)[65].

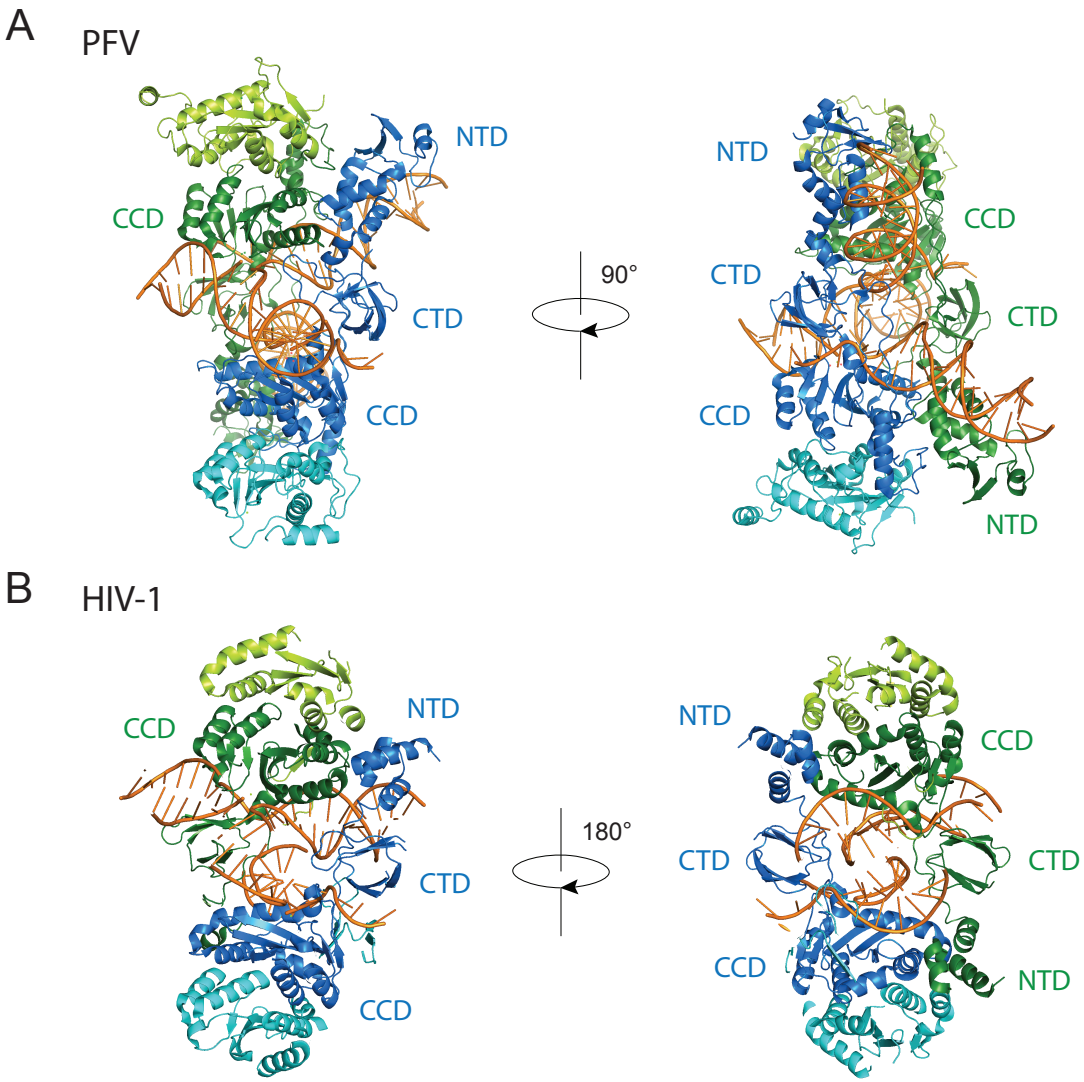

Figure 2: Structures of retroviral intasomes
(A) Structure of the PFV IN intasome complex (pdb code 3os0.) Two IN dimers (green or blue) form a tetramer in complex with viral and host DNA (orange.) The inner monomers (darker shade) interact with DNA while the outer monomers (lighter shade) stabilize the complex. (B) Structure of the HIV-1 IN intasome complex (pdb code 5u1c.) The structure of the IN tetramer is similar to that of other retroviruses.

After forming the intasome complex, IN catalyzes insertion of the viral DNA into the host DNA in two separate steps: 3' processing and strand transfer (**Fig. 3**). During 3' processing IN hydrolyzes a phosphodiester bond at either end of the viral DNA and removes two to three nucleotides in front of an invariant 5'-CA-3' dinucleotide, creating free 3' hydroxyl groups[30,66-68]. Then, during the strand transfer

reaction, the intasome binds the target host DNA and uses the 3' hydroxyls at either end of the viral DNA as nucleophiles to cut the host DNA in a staggered fashion, at the same time joining the viral DNA to the 5' ends of the cut host DNA[69-71]. Finally, the intasome dissembles, leaving loose 5' overhangs on the viral DNA and a pair of single-stranded gaps on either side of the integrated viral DNA which are subsequently repaired by host cell machinery[72]. As a result of integration and subsequent gap repair a short segment of the target DNA sequence is duplicated, and flanks the integrated provirus. The length of the duplicated sequence varies between retroviruses, with HIV-1 generating 5-bp duplications[73,74].

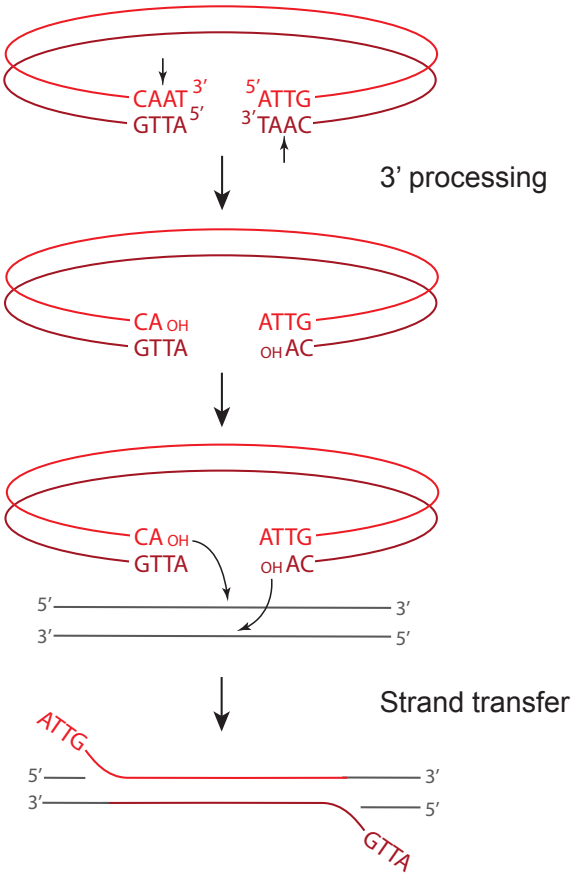

Figure 3: Mechanism of retroviral integration IN catalyzes integration in two steps: 3' processing and strand transfer. During 3' processing IN removes a dinucleotide from the 3' ends of the viral DNA (red) to expose free 3' hydroxyls. During strand transfer IN inserts the 3' ends of the viral DNA into the host DNA (gray), leaving gaps in the target DNA and the loose 5' ends of viral DNA. The gaps and loose ends are subsequentially repaired by host cell machinery.

HIV-1 does not integrate randomly but rather preferentially targets transcriptionally active genes in the nuclear periphery[75-77]. The pre-integration complex (PIC) is guided to its integration site by the chromatin-associated cellular protein lens epithelium-derived growth factor (LEDGF), also called transcriptional coactivator p75, which interacts with IN at its C-terminal integrase-binding domain[78-81].

The LEDGF/p75 N-terminus consists of a PWWP domain, which binds nucleosomes trimethylated at Lys36 of histone 3 (H3K36me3), an epigenetic mark associated with transcriptionally active sites[82,83]. Stringent knockdown or knockout of LEDGF/p75 significantly diminishes HIV-1 titers by specifically inhibiting integration, and also changes integration site-selection[84-86]. Additionally, replacing the PWWP domain of LEDGF/p75 with a heterologous chromatin binding domain redirects HIV-1 integration to chromatin regions bound by the alternative domain[87], further supporting the conclusion that LEDGF/p75 is responsible for guiding and tethering the HIV-1 PIC to its integration site.

Integrase strand-transfer inhibitors (INSTIs) prevent the integration reaction by targeting the strand transfer step[3]. These drugs bind to the active site of the IN CCD, displacing the reactive 3' end of the viral DNA and preventing its insertion into the host DNA[59]. Mutations in the IN active site can confer resistance to INSTIs by directly or indirectly inhibiting drug binding, albeit at a viral fitness cost[15,88,89]. As a result, other compensatory mutations which increase the catalytic activity of IN are additionally found in patients undergoing INSTI therapy[88,89]. Emergence of resistance and cross-resistance is commonly observed for the two first-generation INSTIs, raltegravir and elvitegravir[90]. In spite of the improved potency and higher barriers for resistance, second-generation inhibitors also do select for viral resistance[88,91] highlighting the need for antiretroviral compounds that inhibit IN by a different mode of action.

1.4 Virion morphogenesis

Virion assembly, release, and maturation is a multistep process involving coordinated protein-protein, RNA-RNA, and protein-RNA interactions[92]. Like all retroviruses, HIV-1 selectively packages two copies of full-length vRNA genome[93], which are non-covalently dimerized at their 5' untranslated region (5' UTR). The HIV-1 5' UTR is highly structured and forms six stem-loops with various roles in transcriptional regulation, reverse transcription, dimerization, RNA splicing, and packaging (**Fig. 4**)[94]. The regions responsible for RNA dimerization and packaging overlap, and contains four stem loop structures- SL1, SL2, SL3, and SL4, which are often collectively referred to as the packaging sequence, or psi (ψ). Dimerization of the RNA molecules is required for packaging and infectivity, and is initiated by a region termed the dimer initiation site in SL1. This site contains an apical bulge of nine bases, six of which form a palindrome, allowing the formation of classic Watson-Crick base pairs with the complementary sequence on the other RNA molecule, resulting in a "kissing-loop" structure[42,94-96]. The dimer initiation site is able to mediate dimerization of RNA molecules both *in vitro*[97-100] and i*n vivo*[101-103], and is a major determinant in partner selection and copackaging[101,102].

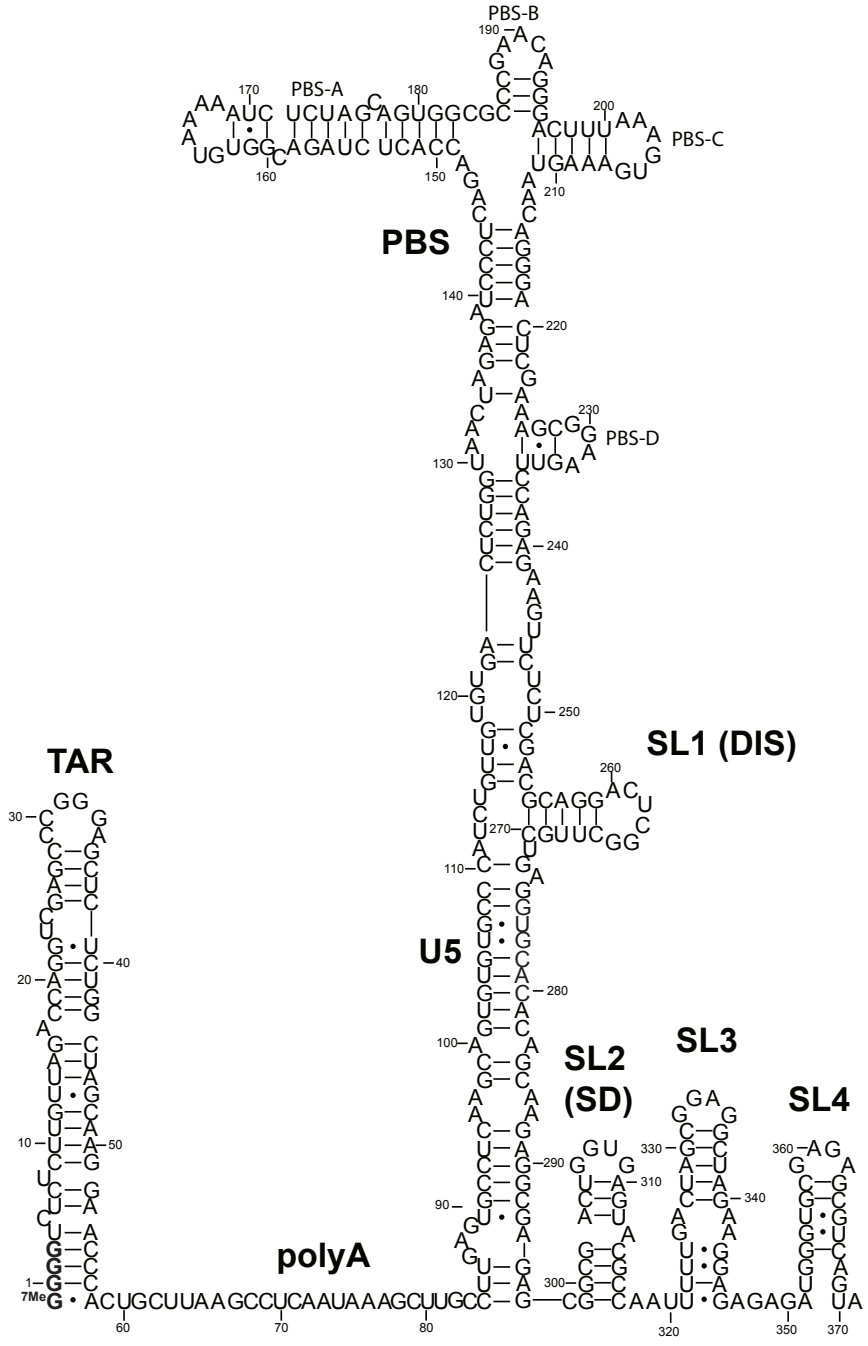

Figure 4: Structure of the HIV-1 RNA 5' UTR
The HIV-1 RNA 5' UTR is highly structured and contains six stem loop structures, including SL1 (dimer initiation site or DIS), SL2 (splice donor site or SD), SL3, and SL4, which are collectively referred to as the packaging sequence or psi (ψ).

Early biochemical studies have found that the viral Gag polyprotein first interacts with vRNA in the cytoplasm as a monomer or low-order multimers, and brings the genome to the plasma membrane[104]. Further Gag molecules are then recruited to the nucleation site and Gag forms high-order multimers through interactions mediated by CA-CA interactions with neighboring Gag molecules. Many of these findings were later corroborated by total internal reflection fluorescence (TIRF) microscopy studies[105-107]. In these experiments, vRNA was observed reaching the plasma membrane first, followed by recruitment of further Gag molecules soon after. In the absence of Gag, vRNA moved rapidly towards and away the plasma membrane, suggesting that Gag is responsible for docking vRNA at the plasma membrane. Over time the amount of Gag at the nucleation site increased, consistent with many Gag molecules polymerizing around the initial Gag-RNA complexes [105-107].

The main contact point with vRNA within Gag is its NC domain, which is later cleaved to form mature NC protein during virion maturation. The RNA 5' UTR SL2 and SL3 structures appear to be recognized by NC, which adopts distinct conformations to bind either stem loop [108,109]. In addition to recognizing structured elements on the HIV-1 RNA, there is also evidence that Gag recognizes dimerized RNA[110]. While a minimal sequence both necessary and sufficient for the packaging of the HIV-1 genome has not been defined, a RNA sequence containing SL1, SL2, and SL3 can both dimerize and bind NC *in vitro*[111], and mutations within the 5' packaging sequence prevent RNA being packaged into viral particles[112]. Likewise, deletion of NC prevents RNA from being packaged and generates particles devoid of the HIV-1 genome [113]. NC binding to RNA is mediated by two CCHC-type zinc knuckle motifs [114-116], and swapping the NC domain of HIV-1 Gag with that of murine leukemia virus (MLV) Gag allows the chimeric HIV-1 Gag protein to package the MLV genome[117,118], further demonstrating the importance of NC in genome packaging. Interestingly, replacing the HIV-1 Gag NC domain with the mouse mammary tumor virus (MMTV) NC domain does not change Gag's preference for packaging HIV-1 RNA[119], suggesting that NC alone does not account for the specificity of HIV-1 genome packaging. Gag-RNA binding is dynamic, and changes as virions assemble, bud, and mature. In the cytosol the Gag NC domain preferentially binds structured elements of the HIV-1 genome and displays a preference for G- and U-rich

elements on cellular mRNAs, while the matrix domain (MA) selectively binds cellular tRNAs[120]. In contrast, during virion assembly at the plasma membrane NC preferentially binds A-rich sequences on the viral genome as well as on cellular mRNAs, while MA dissociates from tRNAs and binds the plasma membrane, facilitating budding of the virion[120].

After assembling at the plasma membrane spherical immature virions bud off from the infected cell **(Fig. 5)**. In immature particles approximately 2000-4000 Gag molecules[121] are radially arranged along the inside of the viral envelope, with MA anchored to the membrane at one end and NC, still bound to vRNA, projecting towards the interior. Immediately after or during budding, the virion undergoes a maturation process in which the viral protease enzyme (PR) cleaves Gag and Gag-Pol at multiple sites in a defined sequence to produce independent viral structural and replicative proteins. Gag is cleaved to produce MA, CA, NC, p6, and two spacer peptides (SP1 and SP2) while Pol is cleaved to yield the viral enzymes, PR, RT, and IN[42,43,122]. The processed proteins then rearrange to form the structure of the mature virion. MA remains associated with the inner side of the viral membrane and forms a discontinuous shell immediately under the membrane. Approximately 1000-1500 monomers of CA assemble to form the capsid lattice[121]. In HIV-1 the capsid takes on a characteristic conical shape, and is composed of approximately 250 hexameric and 12 pentameric rings of CA that are stabilized by interactions within and between subunits[123-128]. Enclosed inside the viral capsid are the two single-stranded HIV-1 RNA molecules bound by NC, and associated with IN and RT, together forming the vRNP[42,43,122].

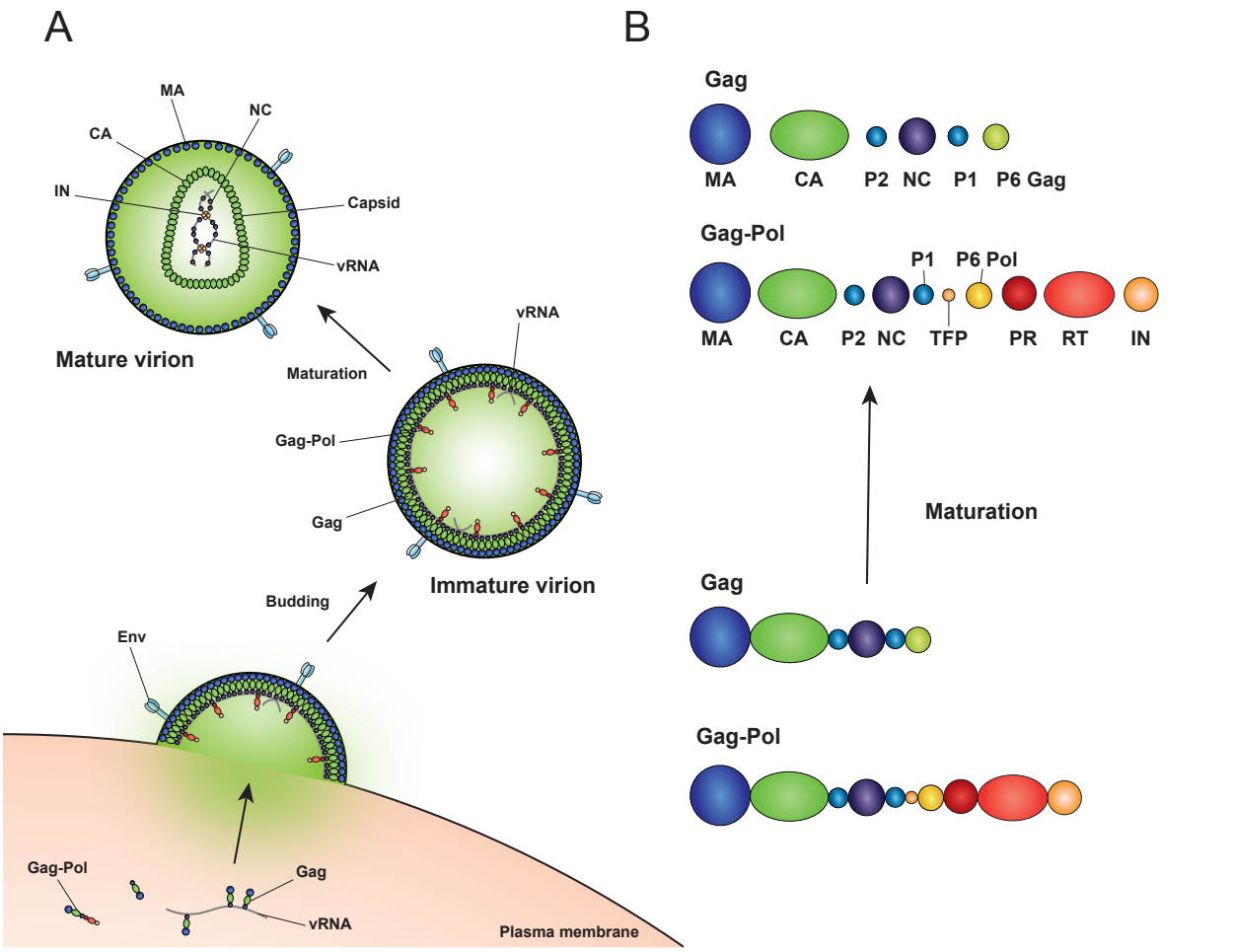

Figure 5: Virion morphogenesis and maturation
(A) The Gag and Gag-Pol polyproteins assemble with vRNA at the plasma membrane, bud from the surface of the cell as immature virions, and then undergo a maturation process. (B) During maturation PR cleaves Gag and Gag-Pol into independent structural and replicative proteins.

Thus, virion morphogenesis is a highly complex process that requires coordinated interaction between the Gag polyprotein and viral RNA, as well as regulated cleavage of Gag into separate mature proteins. While the process has long been thought to be driven solely by Gag, there is emerging evidence that IN plays an unexpected role in proper placement of the viral RNA genome inside the capsid during maturation.

1.5 Integrase in virion maturation

While integration is the canonical function of IN, early mutagenesis studies indicated that IN may also play other roles in virus replication. In particular, a group of IN substitutions referred to as class II IN mutations, lead to pleiotropic effects in HIV-1 replication, including defects in particle assembly[52,129-141], morphogenesis[18,52,131,137-139,142,143] and reverse transcription in target cells[18,51,52,133,135-137,139,141-159], in some cases without impacting IN catalytic function *in vitro*[50,131,132,135,136,145,146,149,151,160,161]. When visualized using electron microscopy, viral particles of class II IN mutant viruses contain vRNP complexes mislocalized outside the capsid lattice [18,52,131,137-139,142,143]. A similar phenotype was noted in IN-deleted viruses[138], again suggesting that IN is necessary for proper virion morphogenesis. Such aberrant viral particles are generally referred to as "eccentric particles," due to the mislocalization of the vRNPs outside the capsid lattice, and are morphologically distinct from immature virions (**Fig. 6**).

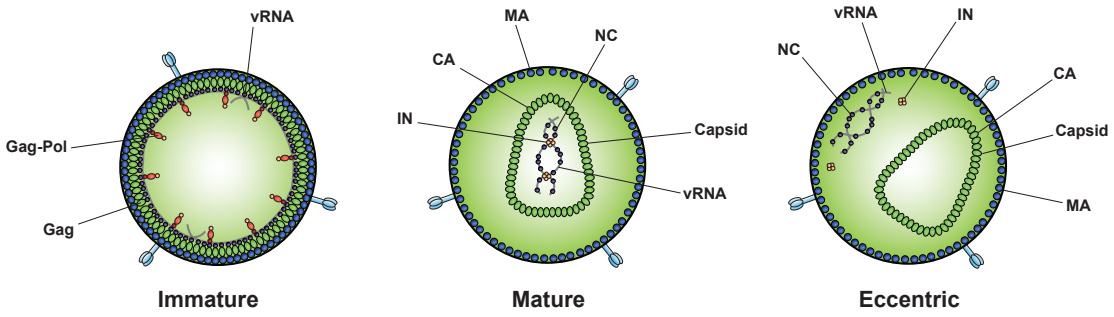

Figure 6: Virion morphologies
Immature viral particles consist of many molecules of Gag and Gag-Pol concentrically arranged along the inner leaflet of the viral membrane and bound to vRNA at the NC domain. In mature viral particles the vRNA is bound by NC and condensed with RT and IN to form the vRNP, which is enclosed in the conical capsid lattice made up of CA monomers. In eccentric viral particles the vRNP is mislocalized outside of the capsid.

Surprisingly, it was recently discovered that treatment of virus producing cells with a class of 2-(quinolin-3-yl) acetic acid derivatives known as allosteric IN inhibitors (ALLINIs) (also called noncatalytic IN inhibitors (NCINIs), lens epithelium-derived growth factor (LEDGF)/p75-IN inhibitors (LEDGINs), IN-LEDGF/p75 allosteric inhibitors (INLAIs), or multimeric IN inhibitors (MINIs)) results in generation of particles with eccentric morphologies[142,143,162,163]. ALLINIs were originally designed to

prevent integration by interfering with IN binding to the cellular cofactor, lens epithelium–derived growth factor (LEDGF/p75), important for targeting the viral preintegration complex to the host chromosome[164]. The compounds compete with LEDGF binding to IN by engaging the V-shaped binding pocket created by the catalytic core domain of two IN dimers in the intasome complex[143,164-169]. In addition to preventing IN-LEDGF interaction, ALLINIs also prevent integration in a LEDGF-independent manner by inducing aberrant IN multimerization, locking IN in catalytically inactive multimers which are unable to assemble on viral DNA and carry out the integration reaction[165,169]. However, subsequent studies found that many ALLINIs are more potent when added to producer cells, and inhibit viral replication at the later stages of the viral life cycle[143,162,163,166-168]. Specifically, treatment with ALLINIs interferes with virion morphogenesis and leads to the generation of eccentric viral particles with vRNPs mislocalized outside the capsid lattice, strikingly similar to those generated by class II IN mutations[143,162,163,167]. Similar to the mechanism by which they can prevent integration, ALLINIs are proposed to interfere with virion morphogenesis by inducing aberrant IN multimerization, and mutations that confer resistance to ALLINIs also prevent ALLINI-induced IN multimerization[162,170]. Many class II IN mutations also alter IN multimerization[62,160,171,172], suggesting that proper multimerization is important for IN's function during virion morphogenesis. However, a defined mechanism by which IN ensures viral RNA is correctly packaged inside the capsid lattice remained elusive for many years.

A seminal study in 2016 revealed that IN binds viral genomic RNA in mature virions, and that IN-RNA binding is necessary for viral replication[18]. Crosslinking immunoprecipitation sequencing (CLIP-seq), an approach that captures protein-RNA interactions in relevant physiological settings[120], was instrumental in this discovery and demonstrated that IN binds the HIV-1 genome at discrete sites with a distinct binding pattern from that of NC. IN not only binds RNA, but also modulates RNA structure in vitro by bridging multiple RNA molecules together[18]. Several basic residues in the IN CTD- K264, K266, and K273- directly interact with RNA, and substitutions at these positions abolish IN-RNA binding in virions. Importantly, virus production in the presence of ALLINIs, BI-D and BI-B2, also prevented IN-RNA binding, likely through aberrant IN multimerization as detailed below[18]. Finally, inhibiting IN

interaction with RNA, either by introducing mutations at the CTD binding site or by ALLINI-treatment, leads to the generation of eccentric, non-infectious viral particles with vRNPs mislocalized outside of the core[18].

1.6 Concluding remarks

HIV-1 IN is a multifunctional protein with an essential role in at least two stages of the viral life cycle. During integration, IN binds both viral and host DNA and orchestrates the insertion of the viral DNA into the host chromosome, fulfilling a critical step in retroviral replication. During virion morphogenesis, IN binds viral RNA and ensures its proper placement within the capsid. Both integration and virion morphogenesis are essential for virus replication, and inhibiting the role of IN in either of these processes is an attractive therapeutic strategy. IN's catalytic function in integration has already been successfully targeted by a number of antiretroviral drugs, but viral mutations that confer resistance to these compounds have been reported in clinical settings. Importantly, because all clinically approved IN inhibitors target the same function of IN, the emergence of resistance mutations can often preclude the use of multiple drugs. Therefore, compounds that target the novel role of IN in virion maturation could be valuable additions to the current antiretroviral arsenal. The discovery that IN-RNA binding is critical to proper placement of viral RNA in virions offers a glimpse into the role IN plays in the late stage of the viral life cycle, but much is still unknown about how IN functions in virion morphogenesis. A better understanding of this function of IN will illuminate an important step in the life cycle of HIV-1, and perhaps other retroviruses, and can inform the development of both new and improved drugs for the treatment of HIV.

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Chapter 2:

Integrase-RNA interactions underscore the critical role of integrase in HIV-1 virion morphogenesis

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Jennifer L. Elliott and Sebla B. Kutluay conceived of and designed experiments. Pratibha C. Koneru performed the biochemical analysis of the multimerization and RNA-biding properties of mutant INs *in vitro* (Figure 4B; Figure 5, Figure 5 – Supplemental Figure 1.) Wen Li performed the EM analysis of virion morphology (Figure 6.) All other work was performed and analyzed by Jennifer L. Elliott.

2.1 ABSTRACT

A large number of human immunodeficiency virus 1 (HIV-1) integrase (IN) alterations, referred to as class II substitutions, exhibit pleotropic effects during virus replication. However, the underlying mechanism for the class II phenotype is not known. Here we demonstrate that all tested class II IN substitutions compromised IN-RNA binding in virions by one of three distinct mechanisms: i) markedly reducing IN levels thus precluding formation of IN complexes with viral RNA; ii) adversely affecting functional IN multimerization and consequently impairing IN binding to viral RNA; iii) directly compromising IN-RNA interactions without substantially affecting IN levels or functional IN multimerization. Inhibition of IN-RNA interactions resulted in mislocalization of the viral ribonucleoprotein complexes outside the capsid lattice, which led to premature degradation of the viral genome and IN in target cells. Collectively, our studies uncover causal mechanisms for the class II phenotype and highlight an essential role of IN-RNA interactions for accurate virion maturation.

2.2 INTRODUCTION

Infectious HIV-1 virions are formed in a multistep process coordinated by interactions between the HIV-1 Gag and Gag-Pol polyproteins, and the viral RNA (vRNA) genome. At the plasma membrane of an infected cell, Gag and Gag-Pol molecules assemble around a vRNA dimer and bud from the cell as a spherical immature virion, in which the Gag proteins are radially arranged[1-3]. As the immature virion buds, the viral protease enzyme is activated and cleaves Gag and Gag-Pol into their constituent domains, triggering virion maturation[1,2]. During maturation the cleaved nucleocapsid (NC) domain of Gag condenses with the RNA genome and *pol*-encoded viral enzymes [reverse transcriptase (RT) and integrase (IN)] inside the conical capsid lattice, composed of the cleaved capsid (CA) protein, which together form the core[1-3].

After infection of a target cell, RT in the confines of the reverse transcription complex (RTC) synthesizes linear double stranded DNA from vRNA[4]. The vDNA is subsequently imported into the nucleus, where the IN enzyme catalyzes its insertion into the host cell chromosome[5,6]. Integration is mediated by the intasome nucleoprotein complex that consists of a multimer of IN engaging both ends of linear vDNA[7]. While the number of IN protomers required for intasome function varies across Retroviridae, single particle cryogenic electron microscopy (cryo-EM) structures of HIV-1 and Maedi-visna virus indicate that lentivirus integration proceeds via respective higher-order dodecamer and hexadecamer IN arrangements[8,9], though a lower-order intasome comprised of an HIV-1 IN tetramer was also resolvable by cryo-EM[9].

A number of IN substitutions which specifically arrest HIV-1 replication at the integration step have been described[10]. These substitutions are grouped into class I to delineate them from a variety of other IN substitutions, which exhibit pleiotropic effects and are collectively referred to as class II substitutions[10-12]. Class II IN substitutions or deletion of entire IN impair proper particle assembly[11,13-25], morphogenesis [11,15,21-23,26-28] and reverse transcription in target cells[10,11,17,19-21,23,25-44], in some cases without impacting IN catalytic function *in vitro*[15,16,19,20,30,31,34,36,45-47]. A hallmark morphological defect of these viruses is the formation of aberrant viral particles with viral ribonucleoprotein (vRNP) complexes mislocalized outside

of the conical CA lattice[11,15,21-23,26-28]. Strikingly similar morphological defects are observed in virions produced from cells treated with allosteric integrase inhibitors (ALLINIs, also known as LEDGINs, NCINIs, INLAIs or MINIs)[26,27,48-55]. ALLINIs induce aberrant IN multimerization in virions by engaging the V-shaped pocket at the IN dimer interface, which also provides a principal binding site for the host integration targeting cofactor lens epithelium-derived growth factor (LEDGF)/p75[50,54,56-60]. The recent discovery that HIV-1 IN binds to the vRNA genome in virions and that inhibiting IN-RNA interactions leads to the formation of eccentric particles provided initial clues about the role of IN during virion morphogenesis[28].

HIV-1 IN consists of three independently folded protein domains: the N-terminal domain (NTD), catalytic core domain (CCD), and C-terminal domain (CTD)[7,61], and vRNA binding is mediated by a constellation of basic residues within the CTD[28]. However, class II IN substitutions are located throughout the entire length of the IN protein[10,12], which raises the question as to how these substitutions impair virus maturation. The structural basis for IN binding to RNA is not yet known; however, in vitro evidence indicates that IN binds RNA as lower-order multimers, and conversely RNA binding may prevent the formation of higher order IN multimers[28]. Notably, aberrant IN multimerization underlies the inhibition of IN-RNA interactions by ALLINIs[28] and subsequent defects in virion maturation[26-28,48,49,51-55]. Therefore, it seems plausible that class II IN substitutions may exert their effect on virus replication by adversely affecting functional IN multimerization. However, a systematical evaluation of the effects of IN substitutions on IN multimerization, IN-RNA binding, and virion morphology is lacking. As such, it remains an open question how functional IN multimerization and/or IN-RNA interactions influence correct virion morphogenesis.

In this work, we aimed to determine the molecular basis of how class II IN substitutions exert their effects on HIV-1 replication. In particular, by detailed characterization of how class II substitutions impact IN multimerization, IN-RNA interactions and virion morphology, we aimed to dissect whether loss of IN binding to vRNA or aberrant IN multimerization underlies the pleiotropic defects observed in viruses

bearing class II IN mutations. Remarkably, we found that class II substitutions either prevented IN binding to the vRNA genome or precluded the formation of IN-vRNA complexes through reducing or eliminating IN from virions. We show that IN tetramers have a strikingly higher affinity towards vRNA than IN monomers or dimers, and a large number of class II IN substitutions inhibited IN binding to RNA indirectly through modulating functional IN tetramerization. In contrast, R262A/R263A and R269A/K273A substitutions within the CTD and the K34A change within the NTD did not perturb IN tetramer formation, and thus likely directly interfered with IN binding to RNA. Irrespective of how IN-RNA binding was inhibited, all class II IN mutant viruses formed eccentric particles with vRNPs mislocalized outside of the CA lattice. Our findings uncover causal mechanisms for the class II phenotype and highlight the essential role of IN-RNA interactions for the formation of correctly matured virions.

2.3 RESULTS

Class II IN substitutions cluster at interfaces that mediate IN multimerization

Substitutions in IN that exhibited a class II phenotype (i.e. assembly, maturation or reverse transcription defects[10-44,62,63] or affected IN multimerization[46,64-67] were selected from past literature (Table 1). Although a structure of IN bound to RNA is not currently available, the location of these substitutions depicted on the model of a tetrameric IN complex (based on cryo-EM structure of the HIV-1 intasome complex consisting of IN and DNA[9]) suggest that the targeted amino acids are positioned at or near monomer-monomer or dimer-dimer interfaces (Figure 1A-B). While not apparent in the tetrameric intasome complex, the CTD mediates IN tetramer-tetramer interactions in the higher-order dodecamer IN structure[9] and has also been shown to mediate IN multimerization in vitro[15].

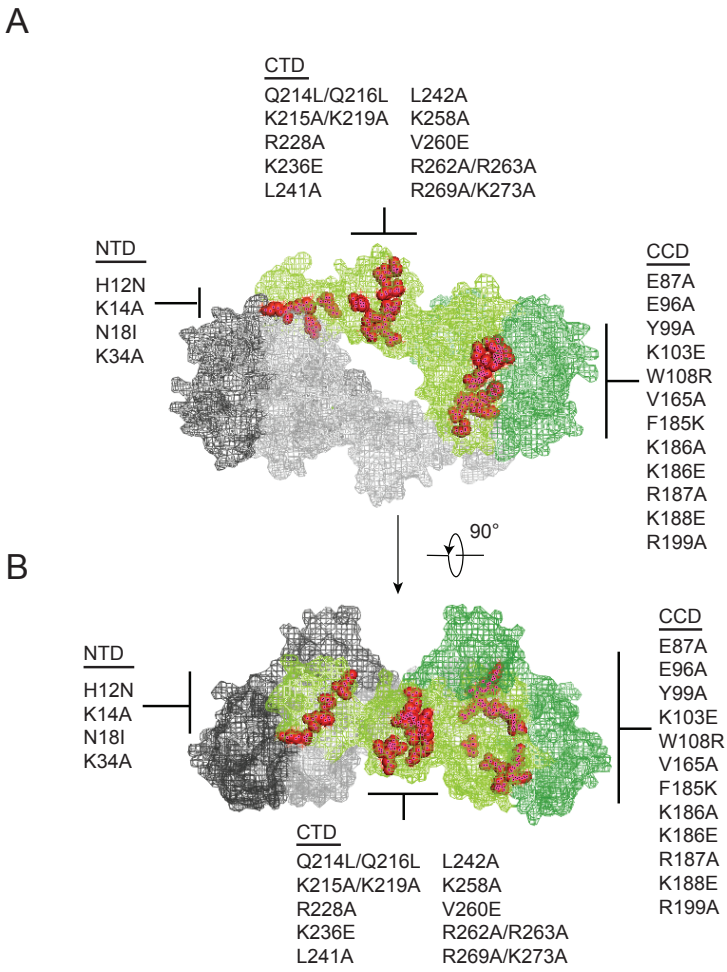

Figure 1. Class II IN substitutions locate throughout IN and cluster at interfaces that mediate IN multimerization. (A) Location of class II IN substitutions used in this study displayed in red on a single IN monomer within the context of the HIV-1 IN tetramer intasome structure consisting of a dimer of dimers (PDB 5U1C). The two dimers are displayed in either gray or green, with individual monomers within each displayed in different shades. The DNA is omitted for clarity. (B) View of the structure displayed in A rotated 90°.

Table 1. Class II IN substitutions used in this study

Substitution	Domain	Remarks
H12N	NTD	• Eccentric virion morphology[11] • IN levels reduced in virions[11] • Multimerization defect *in vitro*[65] • Defective for reverse transcription[19]
K14A		• Multimerization defect *in vitro*[68]
N18I		• IN levels undetectable in virions[69]
K34A		• Defective for reverse transcription[19] • Catalytically active *in vivo*[19]
E87A	CCD	• Multimerization defect *in vitro*[66]
E96A		• Multimerization defect *in vitro*[66]
Y99A		• Multimerization defect *in vitro*[66]
K103E		• IN levels reduced in virions[69]
W108R		• IN levels reduced in virions[69]
V165A		• Eccentric virion morphology[27] • Defective for reverse transcription[20,32] • Catalytically active *in vivo* [19,20,70]
F185K		• Eccentric virion morphology[15] • Multimerization defect *in vitro*[71] • Catalytically active *in vitro*[15]
K186A		• Multimerization defect *in vitro*[68]
K186E		• Catalytic defect *in vitro*[65] • Multimerization defect *in vitro*[65]
R187A		• Multimerization defect *in vitro*[68]
K188E		• Multimerization defect *in vitro*[72]
R199A		• Catalytically active *in vivo*[73]
Q214L/Q216L	CTD	• Defective for reverse transcription[20] • Catalytically active *in vitro*[20]
K215A/K219A		• Defective for reverse transcription[20] • Catalytically active *in vitro* [20]
R228A		• Defective for reverse transcription[19]
K236E		• Defective for reverse transcription[19]
L241A		• Multimerization defect *in vitro*[46] • Catalytic defect *in vitro*[46]
L242A		• Multimerization defect *in vitro* [46] • Catalytic defect *in vitro*[46]
K258A		• Defective for reverse transcription[19]
V260E		• Misfolded protein[46] • Catalytic defect *in vitro*[46]
R262A/R263A		• Defective for reverse transcription[19]
R269A/K273A		• Defective for RNA-binding[74] • Eccentric virion morphology[74] • Defective for reverse transcription[74]

Characterization of the replication defects of class II IN mutant viruses

IN mutations were introduced into the replication competent pNL4-3 molecular clone and HEK293T cells were transfected with the resulting plasmids. Cell lysates and cell-free virions were subsequently analyzed for Gag/Gag-Pol expression, processing, particle release and infectivity. While substitutions in IN had no measurable effect on Gag (Pr55) expression, modest effects on Gag processing in cells was visible for several missense mutant viruses including H12N, N18I, K34A, Y99A, K103E, W108R, F185K, Q214L/Q216L, L242A, V260E, as well as the ΔIN mutant (Figure 2). Nevertheless, particle release was largely similar between WT and IN mutant viruses, as evident by the similar levels of CA protein present in cell culture supernatants (Figure 2, lower panels).

Three distinct phenotypes became apparent by assessing the amount of virion-associated IN and RT enzymes (Figure 2, Figure 2- Figure Supplement 1). First, virion-associated IN was at least 5-fold less than WT with several mutants, including H12N, N18I, K103E, W108R, F185K, L242A, and V260E (Figure 2, Supplemental Table 1). Notably, these substitutions also reduced levels of Gag-Pol processing intermediates in producer cells (Figure 2) and RT in virions (Figure 2, Figure 2- Figure Supplement 1), suggesting that they likely destabilized the Gag-Pol precursor. Near complete lack of processing intermediates with the K14A and N18I substitutions, despite the presence of fully processed RT and IN in virions (detected using a separate polyclonal antibody), is likely due to inaccessibility of epitopes recognized by the monoclonal anti-IN antibody in the processing intermediates. Second, the R228A substitution abolished full-length IN in virions without impacting cell- or virion-associated Gag-Pol levels or processing intermediates; however, a faster migrating species generated by aberrant IN processing and/or IN degradation was visible. A similar but more modest defect was observed for the K34A mutant, which was incorporated into virions at a modestly reduced level alongside a smaller protein species. Third, the remainder of the IN substitutions did not appear to affect IN or Gag-Pol levels in cells or virions.

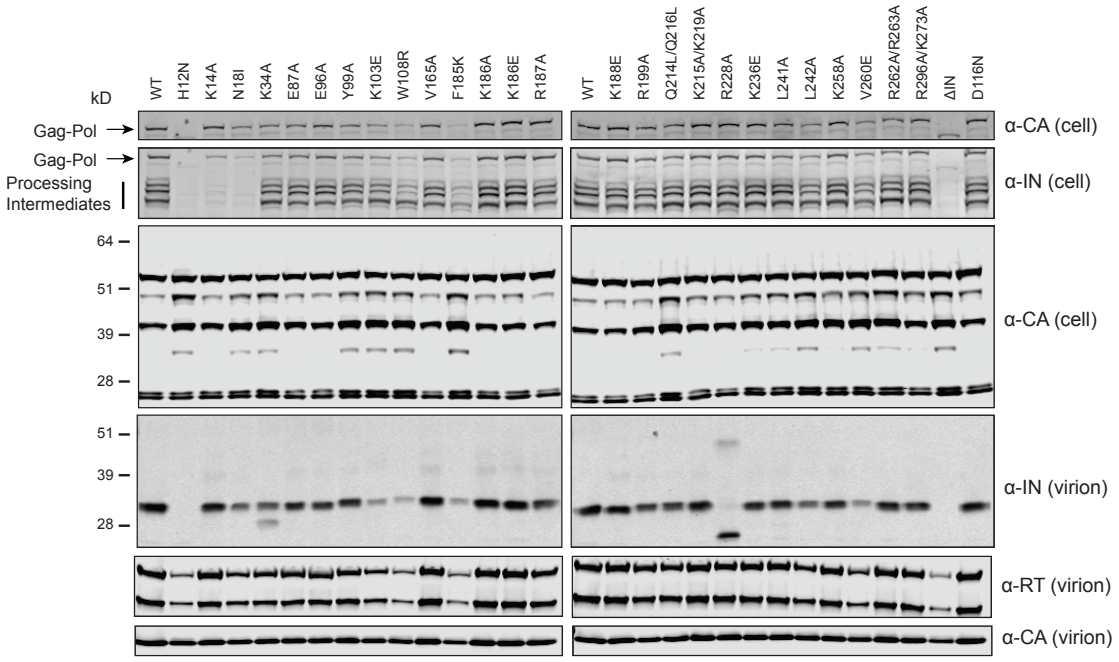

Figure 2. Characterization of the replication defects of class II IN mutant viruses. Immunoblot analysis of Gag and Gag-Pol products in cell lysates and virions. HEK293T cells were transfected with proviral HIV-1_{NL4-3} expression plasmids carrying *pol* mutations encoding for the indicated IN substitutions. Cell lysates and purified virions were harvested two days post transfection and analyzed by immunoblotting for CA, IN and, in the case of virions, RT. Representative image of one of four independent experiments is shown.

Class II IN mutants abolish IN binding to RNA

Using complementary in vitro and CLIP-based approaches, we have previously shown that viral genomic RNAs (vRNA) constitute the primary RNA species bound by IN in virions and that IN interacts with the viral genome through multiple basic residues (i.e. K264, K266, R269, K273) in its CTD[28]. In addition, IN-RNA interactions could also depend on proper IN multimerization, as ALLINI-induced aberrant IN multimerization potently inhibited the ability of IN to bind RNA[28]. Based on this, in the next set of experiments, we aimed to determine whether class II IN mutants bind vRNA, and if not, whether improper IN multimerization may underlie this defect.

IN-vRNA complexes were immunoprecipitated from UV-crosslinked virions and the levels of coimmunoprecipitating vRNA was assessed. Note that substitutions that significantly reduced the amount

of IN in virions (Figure 2, Supplementary Table 1) were excluded from these experiments. All class II IN mutant viruses contained similar levels of vRNA, ruling out any inadvertent effects of the alterations on RNA packaging (Figure 3A). While the catalytically inactive IN D116N bound vRNA at a level that was comparable to the WT, nearly all of the class II IN mutant proteins failed to bind vRNA (Figure 3B). The E96A substitution, which had a fairly modest effect on virus titers as compared to other IN mutants (Chapter 3, Figure 1A), decreased but did not abolish the ability of IN to bind RNA (Figure 3B). Thus, lack of RNA binding ability is a surprisingly common property of a disperse set of class II IN mutants, despite the fact that many of the altered amino acid residues are distally located from the CTD.

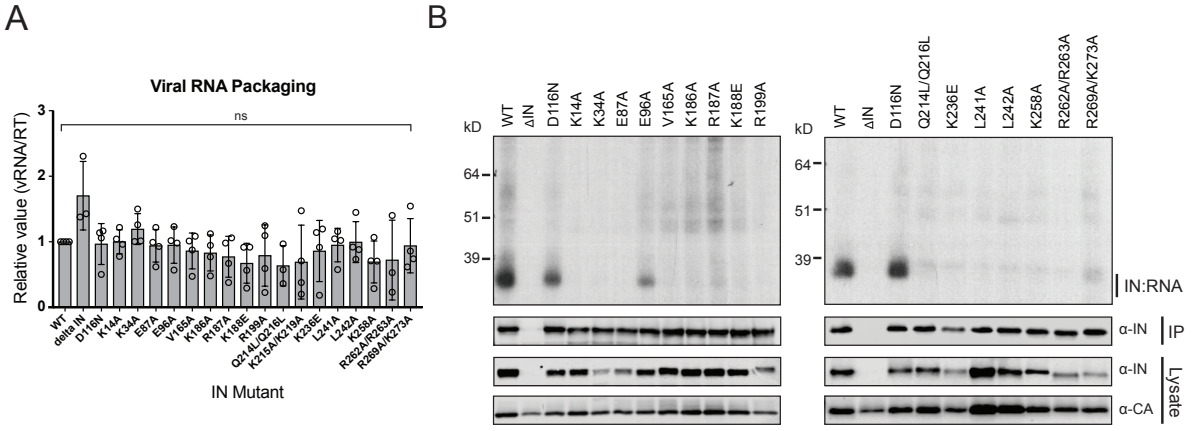

Figure 3. Class II IN substitutions prevent IN binding to the vRNA genome in virions. (A) Analysis of the levels of packaged viral genomic RNA in WT and IN mutant HIV-1_{NL4-3} virions. vRNA extracted from purified virions was measured by Q-PCR. Data was normalized to account for differences in particle yield using an RT activity assay. Normalized quantities of vRNA are expressed relative to WT (set to 1). Columns show the average of three-four independent experiments (open circles) and error bars represent standard deviation (ns, not significant, by one-way ANOVA). (B) Representative autoradiogram of IN-RNA adducts immunoprecipitated from WT or IN mutant HIV-1_{NL4-3} virions. The amount of immunoprecipitated material was normalized such that equivalent levels of WT and mutant IN proteins were loaded on the gel, as also evident in the immunoblots shown below. Levels of IN and CA in input virion lysates is shown in the lower immunoblots. Data is representative of three independent replicates.

IN multimerization plays a key role in RNA binding

As it seemed unlikely that all of the class II IN substitutions directly inhibited IN binding to RNA, we reasoned that they might indirectly abolish binding by perturbing proper IN multimerization. To test

whether class II IN substitutions altered IN multimerization in a relevant setting, purified HIV-1_{NL4-3} virions were treated with ethylene glycol bis (succinimidyl succinate) (EGS) to covalently crosslink IN in situ and virus lysates were analyzed by immunoblotting. IN species that migrated at molecular weights consistent with those of monomers, dimers, trimers and tetramers were readily distinguished in WT virions (Figure 4-Figure Supplement 1). In the majority of the class II mutant particles, IN appeared to exist as monomers as well as higher molecular weight species, representing IN multimers or IN aggregates, with little dimers and no readily detectable tetramers (Figure 4A, Figure 4-Figure Supplement 1). In contrast, K34A, E96A, R262A/R263A and R269A/K273A IN mutants formed dimers and tetramers at similar levels to the WT (Figure 4A, Figure 4-Figure Supplement 1). An undefined smear was present at higher molecular weights for all viruses, possibly as a result of the formation of large IN aggregates upon cross-linking (Figure 4-Figure Supplement 1).

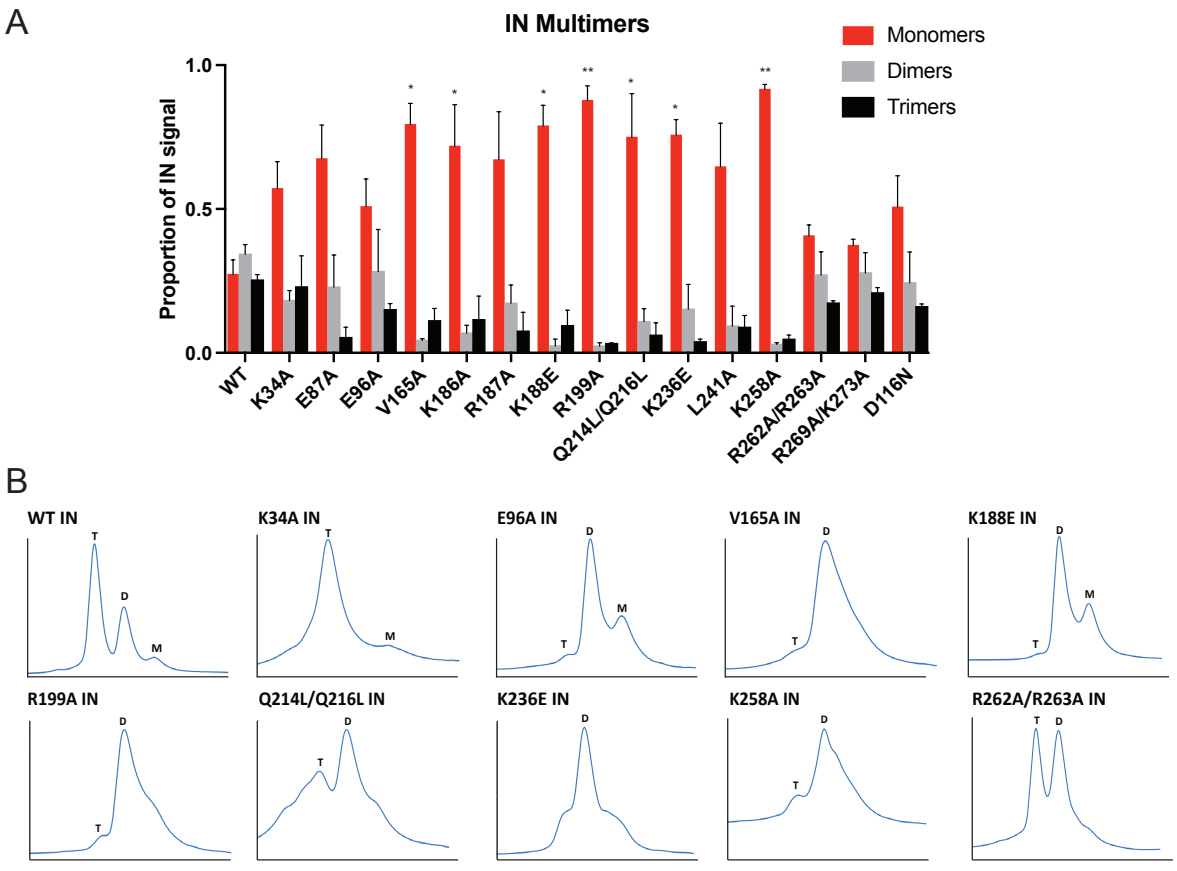

Figure 4. Multimerization properties of class II IN mutants in virions and in vitro. (A) Purified HIV-1 virions were crosslinked with 1mM EGS and analyzed by immunoblotting as detailed in Materials and Methods. IN signal at the molecular weights of 32 kDa (monomers), 64 kDa (dimers), and 96 kDa (trimers) was measured and divided by the total signal of the three multimeric species for each virus. Columns show average of three independent experiments and error bars represent standard error of the mean (**P < 0.05 and *P < 0.01, by one-way ANOVA with Dunnett's multiple comparison test). (B) SEC profiles of 10 μM of WT and indicated IN mutants are shown. X-axis indicates elution volume (mL) and Y-axis indicate the intensity of absorbance (mAU). Tetramers (T), Dimers (D) and Monomers (M) are indicated. Representative chromatograms from two independent analyses are shown.

To corroborate these findings, we analyzed the oligomeric states of recombinant WT, K34A, E96A, V165A, K188E, R199A, Q214L/Q216L, K236E, K258A and R262A/R263A IN proteins by SEC (Figure 4B). Oligomeric states of additional class II IN mutants have previously been characterized *in vitro*[28,46,65,66,68,71,72] and are summarized in Supplementary Table 2. In line with the crosslinking studies in virions, WT, K34A and R262A/R263A INs formed tetramers, while the levels of dimers varied for different mutants. For example, while IN R262A/R263A presented similar levels of tetramers and dimers, IN K34A was primarily tetrameric with a minor dimeric species, as evident by the broad right shoulder of the tetrameric SEC peak (Figure 4B). In contrast, the majority of other IN mutants almost exclusively formed dimers and monomers with little evidence for tetramer formation (Figure 4B). While Q214L/Q216L and K236E IN were predominantly dimeric, the broad base of their chromatograms revealed some evidence for tetramers and monomers as well (Figure 4B).

Next we tested the mutant INs for their ability to bind and bridge cognate RNA oligonucleotides *in vitro*. We have previously shown that recombinant IN binds TAR RNA with a high affinity and provides a nucleation point to bridge and condense RNA[28]. However, IN oligomeric states required for its ability to bind and bridge RNA molecules are not known. We separated WT monomeric, dimeric and tetrameric forms of WT IN by SEC and examined their binding to TAR RNA[28]. Remarkably, while WT IN tetramers bound to TAR RNA with high affinity (2.68 ± 0.16 nM), neither IN dimers nor monomers showed evidence of binding (Figure 5A). In line with this, we found that WT tetramers rather than dimers effectively bridged RNA oligonucleotides *in vitro* (Figure 5B).

We then analyzed a set of class II IN mutants for their ability to bind and bridge TAR RNA *in vitro* (Figure 5C). All class II IN mutants that predominantly formed dimers (Figure 4B) had reduced affinity for RNA compared to WT IN (Figure 5C, Figure 5-Figure Supplement 1B-D). Furthermore, these mutations had even more deleterious effects on the ability of IN to bridge the RNA molecules (Figure 5C). Although IN K34A and IN R262A/R263A could both form tetramers, IN K34A showed a reduced binding affinity for RNA while IN R262A/R263A did not bind RNA at all (Figure 5C, Figure 5-Figure Supplement 1A), suggesting that these residues may be directly involved in IN binding to RNA. Collectively, these results pointed to a key role of IN tetramerization for high affinity binding to RNA and more critically for RNA bridging. Thus, these findings suggest that a defect in proper multimerization underlies the inability of the majority of class II IN mutants to form functional complexes with vRNA.

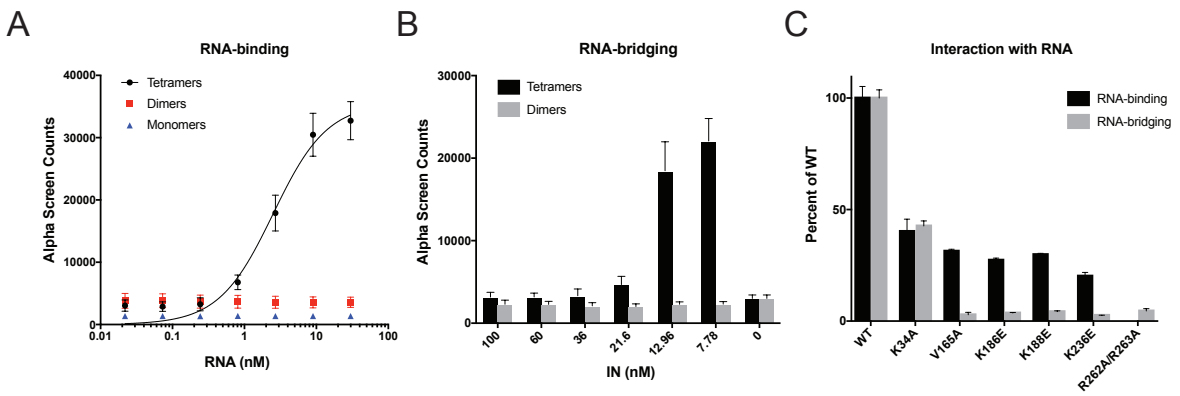

Figure 5. RNA-binding properties of class II IN mutants. (A) Analysis by AlphaScreen assay of 100 nM WT IN monomers, dimers, and tetramers binding to biotinylated TAR RNA after separation by SEC. Graphed data is the average of three independent experiments and error bars indicate standard deviation. (B) Summary of WT IN dimers and tetramers bridging TAR RNA at different protein concentrations as measured by AlphaScreen assay. Graphed data is the average of four independent experiments and error bars indicate standard deviation. (C) Summary of mutant INs binding to TAR RNA (black bars) and bridging TAR RNA (gray bars) compared to WT IN. Percent binding was calculated for each mutant IN by comparing the calculated Kd value to that of WT IN (normalized to 100%) within an experiment. Percent bridging was calculated by comparing the Alpha Counts at 320 nM for each protein to that of WT (normalized to 100%.) Graphed data is average of three independent experiments and error bars represent standard deviation.

Class II IN substitutions generate virions with eccentric morphology

We next sought to determine how preclusion or inhibition of IN-vRNA interactions correlated with particle morphology. Virion morphology of a subset of the IN mutants that inhibited vRNA interactions by three different mechanisms; i.e. those that decreased IN levels in virions (N18I and W108R), those that may have directly inhibited IN binding to RNA (K34A, R262A/R263A), and those that primarily altered IN multimerization (E87A, E96A, F185K, R187A, L241A, L242A), was assessed by transmission electron microscopy (TEM). As expected, the majority of WT particles contained an electron dense condensate representing vRNPs inside the CA lattice, whereas an ΔRT-IN deletion mutant virus produced similar levels of immature particles and eccentric particles (Figure 6A-B). Remarkably, irrespective of how IN-RNA interactions were inhibited, 70-80% of nearly all class II IN mutant particles exhibited an eccentric morphology (Figure 6A-B). Of note, the E96A mutant tended to produce less eccentric and more mature particles than the other IN mutants. Because IN E96A retained partial binding to vRNA in virions (Figure 3B) and partial infectivity (Chapter 3, Figure 1A), we conclude that this infection-deferred mutant harbors a partial class II phenotype.

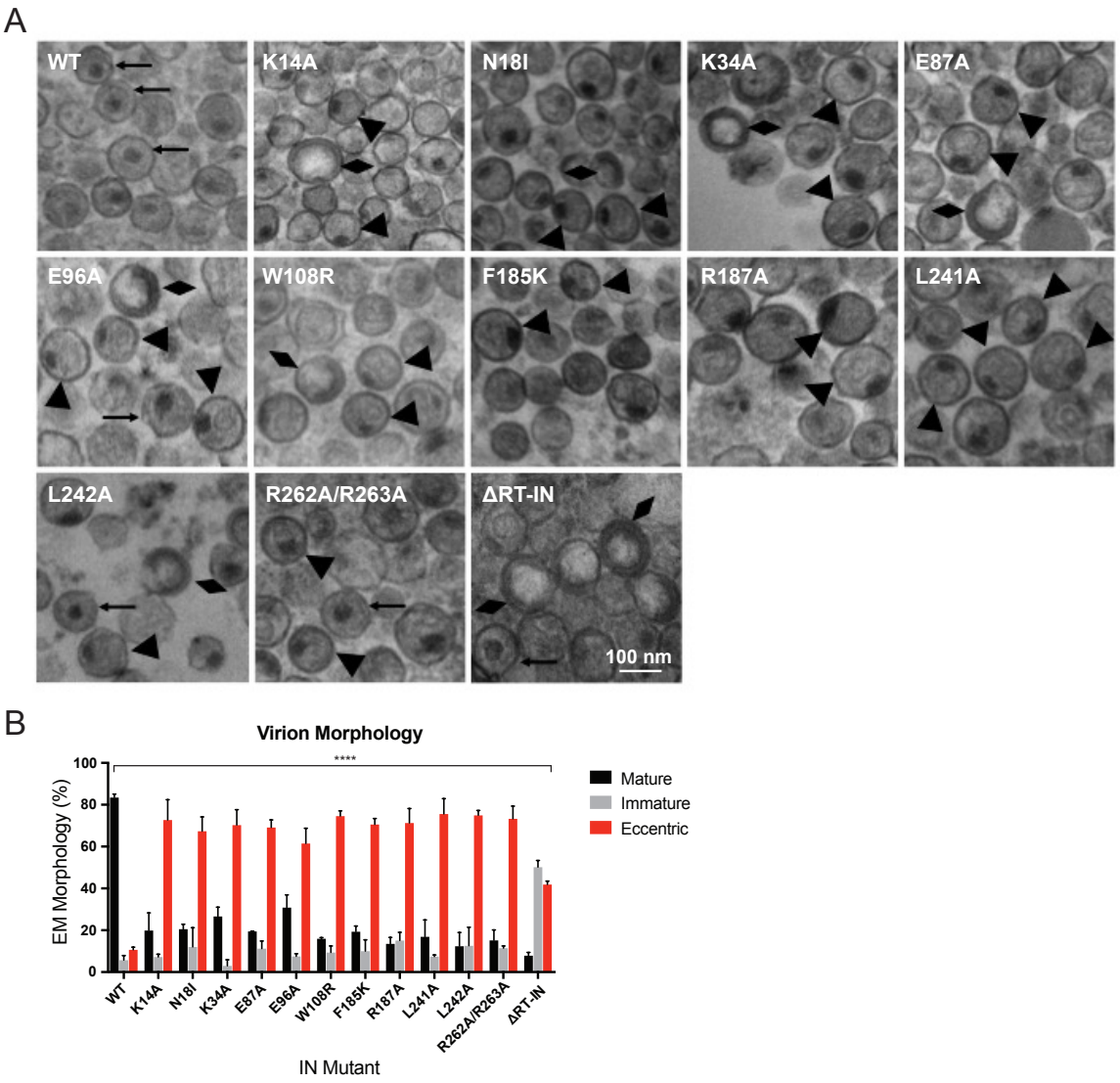

Figure 6. Analysis of class II IN mutant virion morphologies viruses by TEM. (A) Representative TEM images of WT, K14A, N18I, K34A, E87A, E96A, W108R, F185K, R187A, L241A, L242A, R262A/R263A, and ΔRT-IN HIV-1_{NL4-3} virions. Magnification is 30,000x (scale bar, 100 nm). Black arrows indicate mature particles containing conical or round cores with associated electron density; triangles indicate eccentric particles with electron dense material situated between translucent cores and the viral membrane; diamonds indicate immature particles. (B) Quantification of virion morphologies. Columns show the average of two independent experiments (more than 100 particles counted per experiment) and error bars represent standard deviation (****$P < 0.0001$, by repeated measures one-way ANOVA.)

Next, we tested whether inhibition of IN-RNA interactions through class II substitutions changes the localization of IN in virions. The premise for this is based on our previous finding that disruption of IN binding to vRNA through the IN R269A/K273A substitution leads to separation of a fraction of IN from

dense vRNPs and CA containing complexes[76]. Thus, we predicted that inhibition of IN-RNA interactions through the above class II substitutions could lead to a similar outcome. To this end, WT or class II IN mutant virions stripped of the viral lipid envelope by brief detergent treatment were separated on sucrose gradients, and resulting fractions were analyzed for CA, IN, and matrix (MA) content by immunoblotting[76,77]. As before[76], WT IN migrated primarily in dense fractions, whereas the R269A/K273A mutant migrated bimodally (Figure 7A, B). In contrast to our hypothesis, the majority of IN mutants sedimented similarly to WT IN and settled in the denser gradient fractions (Figure 7A, B). Exceptions were the K34A and R262A/R263A IN mutants, a fraction of which migrated in soluble fractions similar to the R269A/K273A mutant, suggesting their localization outside of the capsid lattice. None of the IN substitutions affected the migration pattern of CA (Figure 7C), which distributed bimodally between the soluble and dense fractions, nor the distribution of MA (data not shown), which was found in mainly the soluble fractions. These results suggested that, with the exception of the K34A, R262A/R263A, and R269A/K273A, IN mutant proteins may remain associated with the CA lattice despite inhibition of IN-vRNA interactions. Alternatively, class II IN mutants may localize outside of the CA lattice but form aggregates resulting in a similar migration pattern in dense fractions.

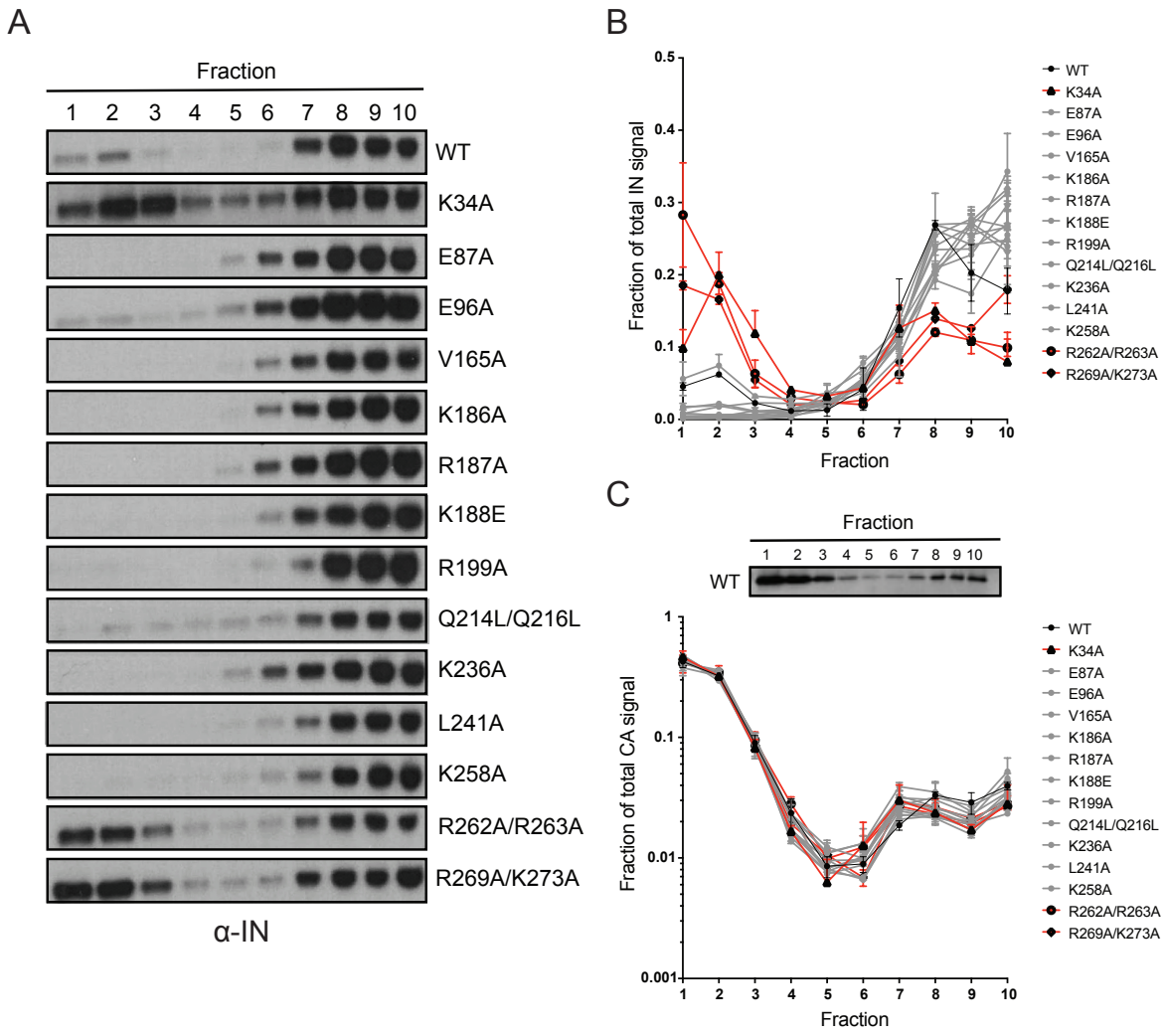

Figure 7. Biochemical analysis of class II IN mutant virus particles. (A) Immunoblot analysis of sedimentation profiles of IN in WT or IN mutant virions. Purified HIV-1_{NLGP} virions were analyzed by equilibrium density centrifugation as detailed in Materials and Methods. Ten fractions collected from the top of the gradients were analyzed by immunoblotting using antibodies against IN. Representative images from one of four independent experiments are shown. (B) Quantitation of IN signal intensity in immunoblots as in (A) are shown. Profile of WT virions is denoted in black, IN mutants that led to bimodal IN distribution are shown in red and others are shown in grey. Graphed data is the average of two independent experiments and error bars indicate the range. (C) Representative immunoblot analysis of sedimentation profile of CA in WT virions and quantitation of CA signal intensity in immunoblots are shown. Profile of WT virions is denoted in black, IN mutants that led to bimodal IN distribution are shown in red and others are shown in grey. Graphed data is the average of two independent experiments and error bars indicate the range.

To test these possibilities, we combined the CA destabilizing K203A substitution, which leads to premature disassembly of the CA lattice *in vitro*[78], with class II IN substitutions K34A, E87A, and

R262A/R263A. Biochemical fractionation of these viruses following membrane stripping as above yielded a similar IN distribution primarily in dense fractions (Figure 7-Figure Supplement 1A). Note that the K203A CA mutation caused the loss of CA in the dense fractions compared to WT viruses, indicating that the CA lattice was indeed destabilized (Figure 7-Figure Supplement 1A). Thus, migration of IN in dense fractions over the gradients does not appear to be dependent on its being enclosed in an intact CA lattice. We then asked if the mutant IN molecules may settle in the dense fractions due to some residual RNA binding not detected by the CLIP assay (Figure 3B). To test this, we introduced the above class II IN substitutions into a Gag-chimeric virus (Gag-bZIP) in which the NC domain is replaced by the leucine zipper domain from the yeast GCN4 protein and as a result is devoid of RNA[79,80]. Of note, this modification additionally destabilized the CA lattice, as indicated by the lack of a second population of CA in dense fractions (Figure 7-Figure Supplement 1B). Lack of RNA packaging and an unstable CA lattice with the Gag-bZIP virus also did not affect the migration pattern of IN over the sucrose gradients (Figure 7-Figure Supplement 1B), nor did presence of RNAse throughout the fractionation experiment (data not shown). The results from these experiments indicate that migration of IN in dense sucrose gradients is independent of its being enclosed in an intact CA lattice and of its binding to vRNA.

2.4 DISCUSSION

Our findings highlight the critical role of IN-vRNA interactions in virion morphogenesis and provide the mechanistic basis for how diverse class II IN substitutions lead to similar morphological defects. We propose that class II IN substitutions lead to the formation of eccentric particles through three distinct mechanisms: (i) depletion of IN from virions thus precluding the formation of IN-vRNA complexes; ii) impairment of functional IN multimerization and as a result, indirect disruption of IN-vRNA binding; iii) direct disruption of IN-vRNA binding without substantially affecting IN levels or its inherent multimerization properties. Irrespective of how IN binding to vRNA is inhibited, all substitutions led to the

formation of eccentric viral particles. Taken together, our findings cement the view that IN binding to RNA accounts for the role of IN in accurate particle maturation.

In regard to the first case (i) above, it was previously shown that IN deletion leads to the formation of eccentric particles[11,27]. Thus, it is reasonable to assume that missense mutations that decreased IN levels in virions phenocopy IN deletion viruses. While it is also possible that these substitutions additionally affected IN binding to vRNA or multimerization, we could not reliably address these possibilities due to the extremely low levels of these proteins in virions.

Our results with different oligomeric states of WT IN show that the tetramers rather than dimers or monomers are able to effectively bind and bridge cognate RNA oligonucleotides (Figure 5A, B). Consistent with these observations, a number of substitutions (V165A, K186E, K188E and K236E) that compromised IN tetramerization also exhibited reduced binding affinity to RNA and failed to bridge separate RNA oligonucleotides (Figure 5C, Figure 5-Figure Supplement 1). More pronounced effects observed with bridging vs. binding assays, suggest a greater importance of IN tetramerization for forming functionally critical IN-RNA condensates. It is possible that IN dimers can weakly bind RNA but they are unable to accommodate and bridge multiple RNA oligonucleotides. In contrast, IN tetramers can both bind with higher affinity and provide larger binding interface to recruit additional RNA molecules. In turn, the ability of IN tetramers to bridge between different segments of viral RNA could be essential for formation of the stable IN-RNA condensates in virions. Consistent with this notion, all class II substitutions that compromised IN tetramerization also failed to form stable IN-RNA complex in virions (Figure 3B). The structural basis for IN binding to RNA is not yet known and it is possible that IN tetramers in the complex with viral RNA differ substantially from those bound to viral DNA within the intasome complex (Figure 1). Nonetheless, our findings indicate the importance of IN tetramers for formation of productive IN-RNA complexes.

Based on MS-based footprinting experiments *in vitro*, we previously found that positively charged residues within the CTD of IN (i.e. K264, K266, K273) directly contact RNA, as was also validated by

CLIP experiments[28]. Our findings here suggest that IN-vRNA contacts may extend to nearby basic residues within the CTD, such as R262 and R263, and perhaps more surprisingly, K34 within the IN NTD, as alterations of these residues did not prevent IN tetramerization (Figure 4A-B) but completely abolished IN-vRNA binding in virions (Figure 3B) and reduced RNA-binding in vitro (Fig 5B-C). This raises the possibility of a second RNA-binding site in the IN NTD. Structural analysis of IN in complex with RNA will be essential to definitively determine how IN binds RNA as well as the precise multimeric species required for binding and bridging/condensation.

The mechanism by which IN-vRNA interactions mediate the encapsidation of vRNPs inside the CA lattice remains unknown. One possibility is that the temporal coordination of proteolytic cleavage events during maturation is influenced by IN-vRNA interactions[81,82]. In this scenario, the assembly of the CA lattice may become out of sync with the compaction of vRNA by NC. Another possibility is that IN-vRNA complexes nucleate the assembly of the CA lattice, perhaps by directly binding to CA. Notably, the biochemical assays performed herein show that class II IN substitutions do not appear to affect the assembly and stability of the CA lattice *in vitro* and in target cells. Although this finding is in disagreement with the previously observed morphological aberrations of the CA lattice present in eccentric particles[26], it is possible that the biochemical experiments used herein lack the level of sensitivity required to quantitatively assess these aberrations or that the cryo-ET procedure impacts the structure of the CA lattice in particular in eccentric virions due to the absence of a packaged vRNP complex. A further possibility is that while the CA lattice in class II IN mutant virions may appear morphologically aberrant, they may still uncoat similar to WT virions in target cells. Further studies deciphering the crosstalk between IN-RNA interactions and CA assembly will be critical to our understanding of the role of IN in accurate virion maturation.

While the mislocalization of the vRNA genome in eccentric particles can be accurately assessed by TEM analysis, precisely where IN is located in eccentric particles remains an open question. Earlier studies based on biochemical separation of core components from detergent-treated IN R269A/K273A virions indicated that IN may also mislocalize outside the CA lattice[76]. In this study, only two class II IN mutants

(K34A and R262A/R263A) demonstrated this phenotype (Figure 7A, B). It is intriguing that the bimodal distribution of IN in this experimental setting was only seen with IN mutants that directly inhibited IN binding to vRNA. A possible explanation for these observations is that improperly multimerized IN is retained within the CA lattice or in association with it. Alternatively, class II IN mutants may localize outside of the CA lattice but form dense aggregates and as a result migrate similar to WT IN. The fact that select class II IN mutants migrate similar to WT IN upon CA destabilization (Figure 7-Figure Supplement 1A) strongly suggests the latter possibility.

Although the basic aspects of virion maturation are conserved, particle morphologies are vastly different across retroviruses[83,84]. It is currently unknown whether IN molecules of other retroviruses regulate viral maturation through binding to vRNAs. Interestingly, mutations within the C-terminus of murine leukemia virus (MLV) IN can similarly cause defects in reverse transcription[85,86], raising the possibility of a conserved role for IN in particle maturation and reverse transcription across retroviruses.

In conclusion, we have identified IN-vRNA binding as the underlying factor for the role of IN in virion morphogenesis. Despite relatively high barriers, drugs that inhibit the catalytic activity of IN do select for resistance, and additional drug classes that inhibit IN activity through novel mechanisms of action would be a valuable addition to currently available treatments. The finding that IN-vRNA interaction can be inhibited in multiple ways- by directly altering residues in the IN CTD or by altering IN multimerization in virions- can help guide the design of future anti-retroviral compounds.

2.5 MATERIALS AND METHODS

Key Reagents

Key Reagents				
Reagent type (species)	**Designation**	**Source or reference**	**Identifiers**	**Additional information**
Gene (human immunodeficiency virus type 1)	Integrase (IN)	NCBI (NC_001802.1)	Gene ID: 155348	
Strain, strain background (*Escherichia coli*)	DH10B	Thermo Fisher Scientific	EC0113	Competent cells
Strain, strain background (*Escherichia coli*)	BL21	Thermo Fisher Scientific	C600003	Competent cells
Cell line (*Homo sapiens*)	HEK293T	ATCC	CRL-11268	
Antibody	Anti-HIV-1 integrase-4 (mouse monoclonal)	Bouyac-Bertoia et al., 2001		WB (1:4000), IP (5µL/100µL of Dyna beads)
Antibody	Anti-HIV-1 integrase-1 (rabbit polyclonal)	This paper		WB (1:1000)
Antibody	Anti-HIV-1 p24 antibody (mouse monoclonal)	NIH AIDS Reagent Program	183-H12-5C	WB (1:100)
Antibody	Anti-HIV-1 reverse transcriptase antibody (rabbit polyclonal)	NIH AIDS Reagent Program	6195	WB (1:1000)
Antibody	Anti-HIV-1 Vpr antibody (rabbit polyclonal)	NIH AIDS Reagent Program	11836	WB (1:1000)
Antibody	Anti-HIV-1 MA antibody (rabbit polyclonal)	NIH AIDS Reagent Program	4811	WB (1:1000)
Commercial assay, kit	QuikChange Site-Directed Mutagenesis Kit	Agilent Technologies	Cat# 200519	
Chemical compound, drug	Ethylene glycol bis(succinimidyl succinate) (EGS)	Thermo Fisher Scientific		

Plasmids

The pNLGP plasmid consisting of the HIV-1_{NL4-3} -derived Gag-Pol sequence inserted into the pCR/V1 plasmid backbone[87] and the CCGW vector genome plasmid carrying a GFP reporter under the control of the CMV promoter[88,89] were previously described. The pLR2P-vprIN plasmid expressing a Vpr-IN fusion protein has also been previously described[75]. Mutations in the IN coding sequence were introduced into both the pNLGP plasmid and the HIV-1_{NL4-3} full-length proviral plasmid (pNL4-3) by overlap extension PCR. Briefly, forward and reverse primers containing IN mutations in the *pol* reading frame were used in PCR reactions with antisense and sense outer primers containing unique restriction endonuclease sites (AgeI-sense, NotI-antisense for NLGP and AgeI-sense, EcoRI-antisense for pNL4-3), respectively. The resulting fragments containing the desired mutations were mixed at 1:1 ratio and overlapped subsequently using the sense and antisense primer pairs. The resulting fragments were digested with the corresponding restriction endonucleases and cloned into pNLGP and pNL4-3 plasmids. IN mutations were introduced into the pLR2P-vprIN plasmid using the QuickChange Site-Directed Mutagenesis kit (Agilent Technologies). Presence of the desired mutations and absence of unwanted secondary changes were verified by Sanger sequencing. HIV-1 CA K203A substitution, which destabilizes the CA lattice[78], was cloned into HIV-1_{NL4-3} bearing class II IN mutations by conventional cloning. Generation of Gag-bZIP chimeras bearing the leucine zipper domain from the yeast GCN4 protein in place of NC, which facilitates Gag dimerization but does not bind RNA has been described before[79,80]. A version of this chimera was generated by replacing NC with a NotI restriction site and cloning of the PCR-amplified GCN4-bZIP in its place. Class II IN mutants were subsequently cloned into this backbone by the QuickChange Site-Directed Mutagenesis kit (Agilent Technologies).

Cells and viruses

All cell lines were originally obtained from American Type Culture Collection and NIH AIDS Reagents where STR profiling was performed. All cell lines are regularly checked for mycoplasma contamination using the MycoAlert mycoplasma detection kit (Lonza) and verified to be free of contamination during the

course of these studies. HEK293T cells (ATCC CRL-11268) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Single-cycle GFP reporter viruses pseudotyped with vesicular stomatitis virus G protein (VSV-G) were produced by transfection of HEK293T cells with pNLGP-derived plasmids, the CCGW vector genome carrying GFP, and VSV-G expression plasmid at a ratio of 5:5:1, respectively, using polyethyleneimine (PolySciences, Warrington, PA). Full-length viruses pseudotyped with VSV-G were produced by transfecting HEK293T cells with the pNL4-3-derived plasmids and VSV-G plasmid at a ratio of 4:1 (pNL4-3:VSV-G).

Immunoblotting

Viral and cell lysates were resuspended in sodium dodecyl sulfate (SDS) sample buffer and separated by electrophoresis on Bolt 4-12% Bis-Tris Plus gels (Life Technologies), blotted onto nitrocellulose membranes and probed overnight at 4°C with the following antibodies in Odyssey Blocking Buffer (LI-COR): mouse monoclonal anti-HIV p24 antibody (183-H12-5C, NIH AIDS reagents), mouse monoclonal anti-HIV integrase antibody[70], rabbit polyclonal anti-HIV integrase antibody raised in-house against Q44-LKGEAMHGQVD-C56 peptide and hence unlikely to be affected by the substitutions introduced into IN in this study, rabbit polyclonal anti-HIV-1 reverse transcriptase antibody (6195, NIH AIDS reagents), rabbit polyclonal anti-Vpr antibody (11836, NIH AIDS Reagents), rabbit polyclonal anti-MA antibody (4811, NIH AIDS Reagents). Membranes were probed with fluorophore-conjugated secondary antibodies (LI-COR) and scanned using a LI-COR Odyssey system. IN and CA levels in virions were quantified using Image Studio software (LI-COR). Alternatively, antibody incubations were done using 5% non-fat dry milk and membranes were probed with HRP-conjugated secondary antibodies and developed using SuperSignal™ West Femto reagent (Thermo-Fisher).

CLIP experiments

CLIP experiments were conducted as previously described[28,91,92]. Cell-free HIV-1 virions were isolated from transfected HEK293T cells. Briefly, cells in 15-cm cell culture plates were transfected with 30 μg

full-length proviral plasmid (pNL4-3) DNA containing the WT sequence or indicated *pol* mutations within the IN coding sequence. Cells were grown in the presence of 4-thiouridine for 16 hr prior to virus harvest. Two days post transfection cell culture supernatants were collected and filtered through 0.22 μm filters and pelleted by ultracentrifugation through a 20% sucrose cushion using a Beckman SW32-Ti rotor at 28,000 rpm for 1.5 hr at 4°C. Virus pellets were resuspended in phosphate-buffered saline (PBS) and UV-crosslinked. Following lysis in RIPA buffer, IN-RNA complexes were immunoprecipitated using a mouse monoclonal anti-IN antibody[70]. Bound RNA was end-labeled with γ-^{32}P-ATP and T4 polynucleotide kinase. The isolated protein-RNA complexes were separated by SDS-PAGE, transferred to nitrocellulose membranes and exposed to autoradiography films to visualize RNA. Lysates and immunoprecipitates were also analyzed by immunoblotting using antibodies against IN.

IN multimerization in virions

HEK293T cells grown on 10-cm dishes were transfected with 10 μg pNL4-3 plasmid DNA containing the WT sequence or indicated *pol* mutations within IN coding sequence. Two days post-transfection cell-free virions collected from cell culture supernatants were pelleted by ultracentrifugation through a 20% sucrose cushion using a Beckman SW41-Ti rotor at 28,000 rpm for 1.5 hr at 4°C. Pelleted virions were resuspended in 1X PBS and treated with ethylene glycol bis(succinimidyl succinate) (EGS) (ThermoFisher Scientific), a membrane permeable crosslinker, at a concentration of 1 mM for 30 min at room temperature. Crosslinking was stopped by addition of SDS sample buffer. Samples were subsequently separated on 3-8% Tris-acetate gels and analyzed by immunoblotting using a mouse monoclonal anti-IN antibody[70].

Size exclusion chromatography (SEC)

All of the mutations were introduced into a plasmid backbone expressing His_6 tagged pNL4-3-derived IN by QuikChange site directed mutagenesis kit (Agilent)[60]. His_6 tagged recombinant pNL4-3 WT and mutant INs were expressed in BL21 (DE3) *E. coli* cells followed by nickel and heparin column purification as described previously[60,93]. Recombinant WT and mutant INs were analyzed on Superdex 200 10/300 GL

column (GE Healthcare) with running buffer containing 20 mM HEPES (pH 7.5), 1 M NaCl, 10% glycerol and 5 mM BME at 0.3 mL/min flow rate. The proteins were diluted to 10 μM with the running buffer and incubated for 1 hr at 4°C followed by centrifugation at 10,000g for 10 min. Multimeric form determination was based on the standards including bovine thyroglobulin (670,000 Da), bovine gamma-globulin (158,000 Da), chicken ovalbumin (44,000 Da), horse myoglobin (17,000 Da) and vitamin B12 (1,350 Da).

Analysis of IN-RNA binding in vitro

Following SEC of IN as above, individual fractions of tetramer, dimer and monomer forms were collected and their binding to TAR RNA was analyzed by an Alpha screen assay as described previously[28]. Briefly, 100 nM His_6 tagged IN fractions (tetramer, dimer and monomer) were incubated with nickel acceptor beads while increasing concentrations of biotinylated-TAR RNA was incubated with streptavidin donor beads in buffer containing 100 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 1 mg/mL BSA, 25 mM Tris (pH 7.4). Followed by 2-h incubation at 4°C, they were mixed and the reading was taken after 1 hr incubation at 4°C by PerkinElmer Life Sciences Enspire multimode plate reader. The Kd values were calculated using OriginLab software.

AlphaScreen-based RNA bridging assays

The RNA bridging property of IN was analyzed by AlphaScreen-based assay as described[28]. Briefly, equal concentrations (1 nM) of two synthetic TAR RNA oligonucleotides labeled either with biotin or DIG were mixed and then streptavidin donor and anti-DIG acceptor beads at 0.02 mg/mL concentration were supplied in a buffer containing 100 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 1 mg/mL BSA and 25 mM Tris (pH 7.4). After 2 hr incubation at 4 °C, indicated concentrations of IN were added to the reaction mixture and incubated further for 1.5 hr at 4 °C. AlphaScreen signals were recorded with a PerkinElmer Life Sciences Enspire multimode plate reader.

Virus production and transmission electron microscopy

Cell-free HIV-1 virions were isolated from transfected HEK293T cells. Briefly, cells grown in two 15-cm cell culture plates (10^7 cells per dish) were transfected with 30 μg full-length proviral plasmid (pNL4-3) DNA containing the WT sequence or indicated *pol* mutations within IN coding sequence using PolyJet DNA transfection reagent as recommended by the manufacturer (SignaGen Laboratories). Two days after transfection, cell culture supernatants were filtered through 0.22 μm filters and pelleted by ultracentrifugation using a Beckman SW32-Ti rotor at 26,000 rpm for 2 hr at 4 °C. Fixative (2.5% glutaraldehyde, 1.25% paraformaldehyde, 0.03% picric acid, 0.1 M sodium cacodylate, pH 7.4) was gently added to resulting pellets, and samples were incubated overnight at 4 °C. The following steps were conducted at the Harvard Medical School Electron Microscopy core facility. Samples were washed with 0.1 M sodium cacodylate, pH 7.4 and postfixed with 1% osmium tetroxide /1.5% potassium ferrocyanide for 1 h, washed twice with water, once with maleate buffer (MB), and incubated in 1% uranyl acetate in MB for 1 h. Samples washed twice with water were dehydrated in ethanol by subsequent 10 minute incubations with 50%, 70%, 90%, and then twice with 100%. The samples were then placed in propyleneoxide for 1 hr and infiltrated overnight in a 1:1 mixture of propyleneoxide and TAAB Epon (Marivac Canada Inc.). The following day the samples were embedded in TAAB Epon and polymerized at 60 °C for 48 h. Ultrathin sections (about 60 nm) were cut on a Reichert Ultracut-S microtome, transferred to copper grids stained with lead citrate, and examined in a JEOL 1200EX transmission electron microscope with images recorded on an AMT 2k CCD camera. Images were captured at 30,000x magnification, and over 100 viral particles per sample were counted by visual inspection.

Equilibrium density sedimentation of virion core components in vitro

Equilibrium density sedimentation of virion core components was performed as previously described[76]. Briefly, HEK293T cells grown in 10-cm cell culture plates were transfected with 10 μg pNLGP plasmid

DNA containing the WT sequence or indicated *pol* mutations within IN coding sequence. Two days post-transfection cell-free virions collected from cell culture supernatants were pelleted by ultracentrifugation through a 20% sucrose cushion using a Beckman SW41-Ti rotor at 28,000 rpm for 1.5 hr at 4 °C. Pelleted viral-like particles were resuspended in PBS and treated with 0.5% Triton X-100 for 2 min at room temperature. Immediately after, samples were layered on top of 30-70% linear sucrose gradients prepared in 1X STE buffer (100 mM NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA) and ultracentrifuged using a Beckman SW55-Ti rotor at 28,500 rpm for 16 hr at 4 °C. Fractions (500 μL) collected from the top of the gradients were analyzed for IN, CA, and MA by immunoblotting as detailed above.

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2.7 SUPPLEMENTAL FIGURES AND TABLES

Supplemental Table 1: Quantitation of IN in virions as measured by western blotting

IN mutant	IN signal (%)	SD	IN mutant	IN signal (%)	SD
WT	100	N/A	K186E	63.2	16.4
H12N	ND	N/A	R187A	34.4	24.5
K14A	40.5	10.3	K188E	45.8	26.3
N18I	16.7	3.1	R199A	41.1	19.2
K34A	32.6	6.9	Q214L/Q216L	31.7	6.3
E87A	31.4	16.1	K215A/K219A	47.7	20.4
E96A	38.4	31.2	R228A	50.2	26.4
Y99A	22.0	10.3	K236E	37.4	14.0
K103E	6.8	2.3	L241A	41.4	12.4
W108R	2.7	0.3	L242A	13.7	3.5
V165A	55.6	32.8	K258A	36.7	19.7
F185K	2.5	1.6	V260E	6.0	4.0
K186A	69.4	27.9	R262A/R263A	52.9	7.0

For each experiment IN signal was normalized to CA signal for each virus, and the resulting value compared to that of WT (set at 100%.) Reported values are the average value (as percent of WT) and standard deviation (SD) between 4 independent experiments. Mutants with less than 20% IN signal of WT are highlighted in gray.

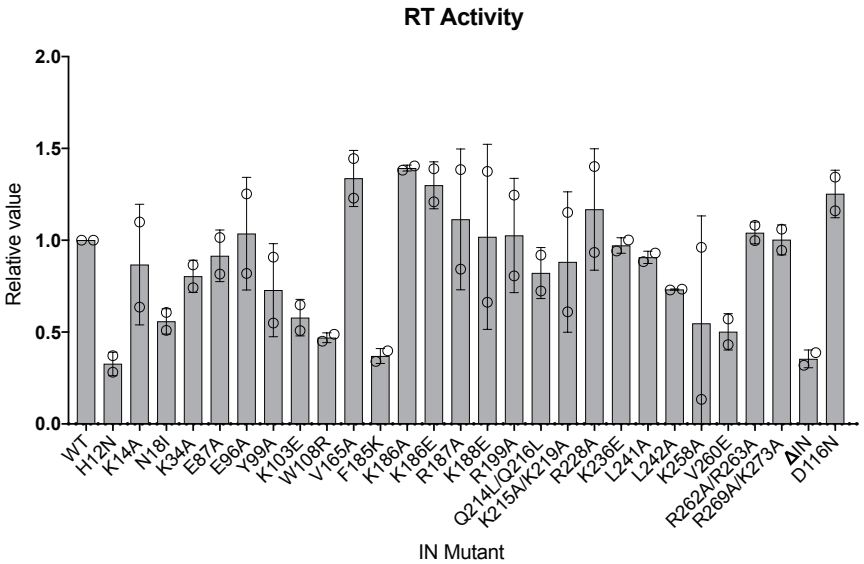

Figure 2-Figure Supplement 1. Characterization of the replication defects of class II IN mutant viruses. (A) Reverse-transcriptase activity measured in HIV-1_{NL4-3} virion lysates. For each repetition RT activities for the IN mutants are expressed relative to the WT (set to 1.) Columns show average of two independent experiments (open circles) and error bars represent standard deviation. (B) Representative immunoblot analysis of Vpr-IN fusion constructs in cell lysates. HEK293T cells were co-transfected with the HIV-1_{NL4-3} IN_{D116N} proviral plasmid along with Vpr-IN expression plasmids encoding for the indicated IN substitutions or an empty vector control. Expression of Vpr-IN constructs in cell lysates was detected using an anti-IN antibody.

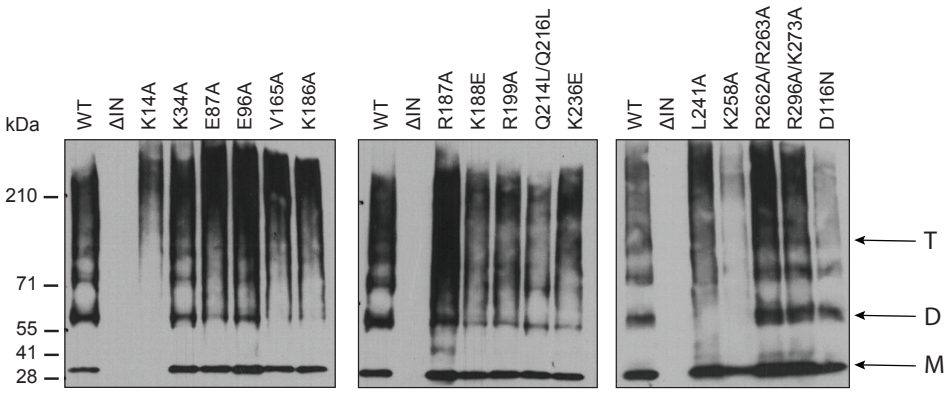

Figure 4-Figure Supplement 1. Multimerization properties of class II IN mutants. Immunoblot analyses of IN multimers in virions. Purified WT or IN mutant HIV-1_{NL4-3} virions were treated with 1 mM EGS, and virus lysates analyzed by immunoblotting using antibodies against IN following separation on 6% Tris-acetate gels. Position of monomers (M), dimers (D), and tetramers (T) are indicated by arrows. Representative image of one of three independent experiments is shown.

Supplementary Table 2: Predominant multimeric species of mutant INs in vitro as assessed by SEC.

IN mutant	Multimeric species	Reference
H12N	Dimer or dimer/monomer mixture	Hare et al., *PLoS Pathog*, 2009
K14A	Dimer	McKee et. al., *J Biol Chem*, 2008
E87A	Dimer	Kessl et al., *Mol Pharmacol*, 2009
F185K	Dimer	Pandey et. al., Biochem, 2011
K186A	Dimer	McKee et. al., *J Biol Chem*, 2008; Houwer et. al., *J Biol Chem*, 2012
K186E	Dimer or dimer/monomer mixture	Hare et al., *PLoS Pathog, 2009*
R187A	Dimer	McKee et. al., *J Biol Chem*, 2008; Houwer et. al., *J Biol Chem*, 2012
L241A	Dimer	Luztke and Plasterk, *J Vir*, 1998
L242A	Dimer/tetramer mixture, shifted to dimer	Luztke and Plasterk, *J Vir*, 1998
R269A/K273A	Tetramers and monomers	Kessl et. al., *Cell*, 2016

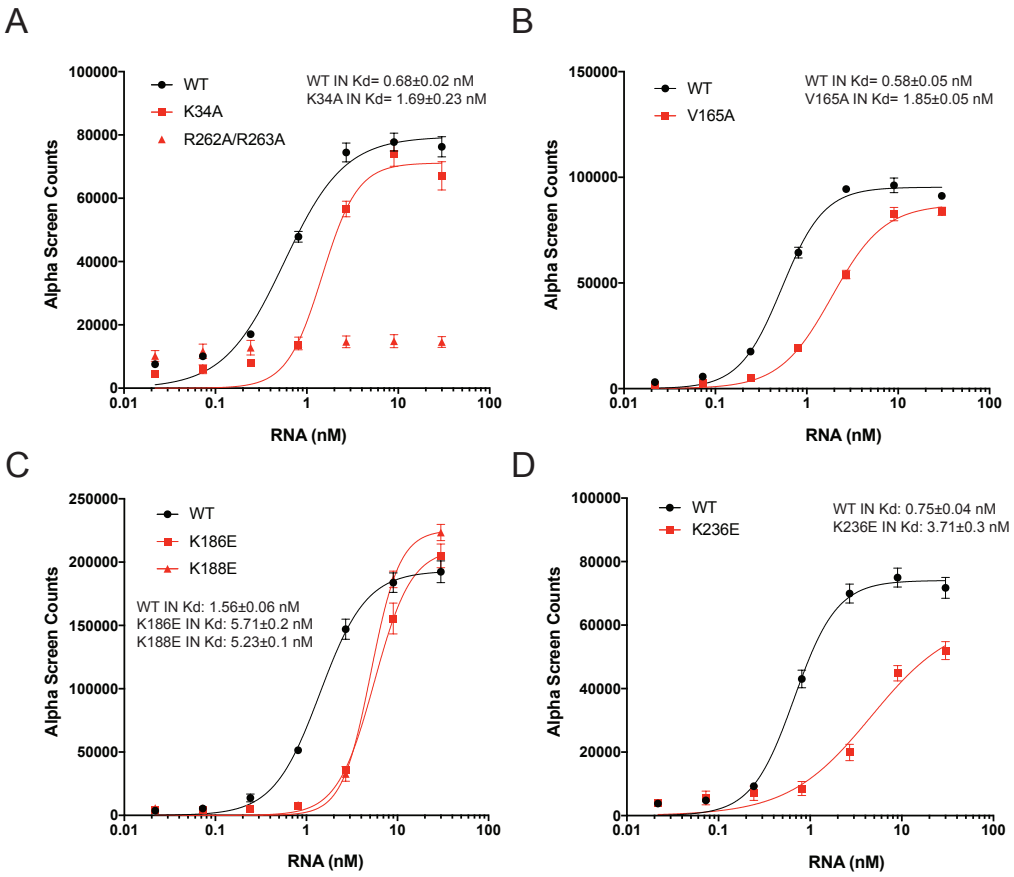

Figure 5-Figure Supplement 1. RNA-binding properties of Class II IN mutants in vitro. (A-D) Analysis of 100 nM WT or mutant INs binding to biotinylated TAR RNA by AlphaScreen assay. Graphed data is the average of three independent experiments and error bars indicate standard deviation.

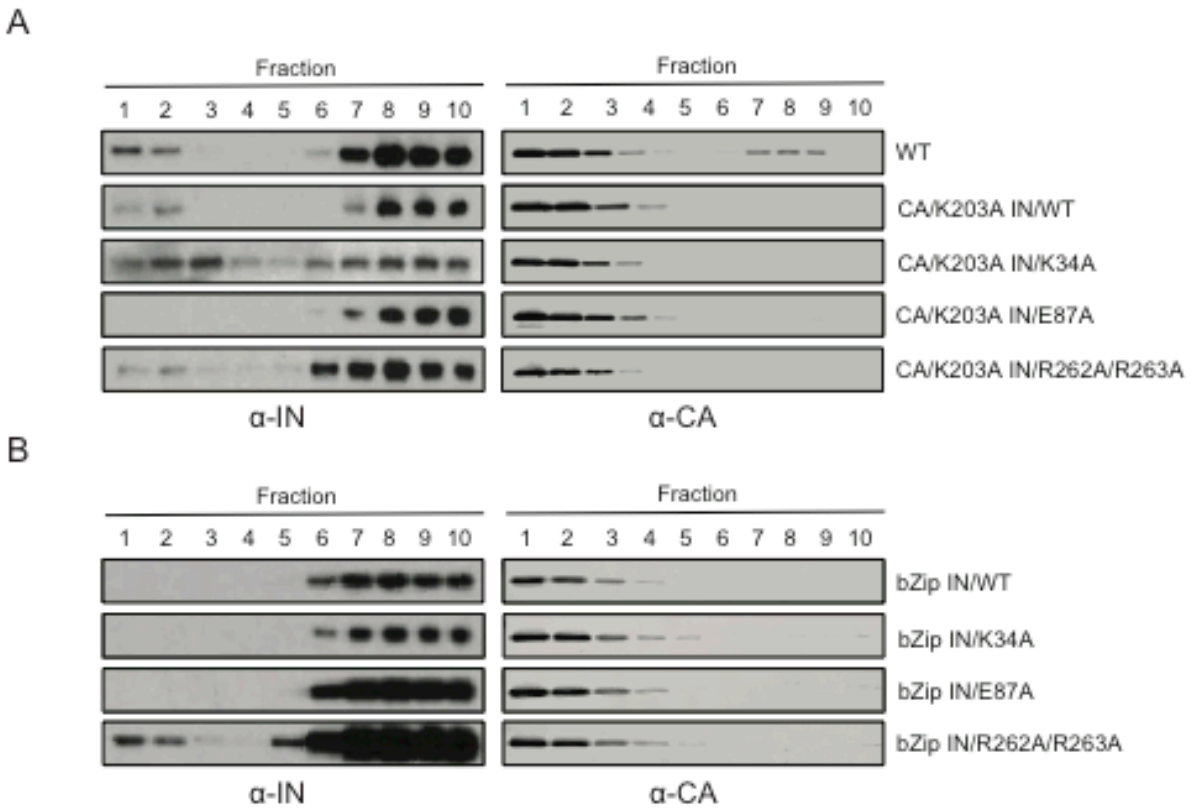

Figure 7-Figure Supplement 1. Biochemical properties of Class II IN mutants in virions upon CA destabilization and in the absence of NC. (A) Immunoblot analysis of sedimentation profiles of IN in HIV-1_{NLGP} CA_{K203A} mutant virions. In addition to the CA_{K203A} mutation, class II mutations were introduced in IN. Purified virions were stripped of the viral envelope using 0.5% Triton X-100 and analyzed by equilibrium density centrifugation as detailed in Materials and Methods. Ten fractions collected from the top of the gradients were analyzed by immunoblotting using antibodies against IN or CA. Representative images from one of two independent experiments are shown. (B) Immunoblot analysis of sedimentation profiles of WT vs. class II IN mutants from HIV-1_{NL4-3} bZIP virions. Purified virions were analyzed by equilibrium density centrifugation, ten fractions were collected from the top of the gradients and subsequently analyzed by immunoblotting using antibodies against IN or CA. Representative images from one of two independent experiments are shown.

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Chapter 3:

The fate of eccentric viral particles

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3.1 ABSTRACT

Mutations in human immunodeficiency virus 1 (HIV-1) integrase (IN) can have a wide range of effects on virus replication. Class II substitutions are defined as those that inhibit the viral life cycle at steps other than integration, and often block virus replication either at or prior to reverse transcription. However, the mechanism for this defect has not been elucidated. Here we demonstrate that all tested class II IN substitutions lead to premature degradation of the viral genome and IN in target cells, likely as a result of the mislocalization of the genome and IN outside the viral capsid. Our findings provide an explanation for why eccentric particles are unable to complete the HIV-1 life cycle.

3.2 INTRODUCTION

In mature HIV-1 virions the two copies of single-stranded RNA genome are condensed and coated by the nucleocapsid protein (NC), together forming the viral ribonucleoprotein complex (vRNP.) The vRNP and viral replicative enzymes reverse transcriptase (RT) and integrase (IN) are enclosed inside of a conical capsid lattice made up of many monomers of capsid (CA) protein, which is released into the cytoplasm of the target cell during viral entry[1-3]. After entry, the single-stranded viral RNA (vRNA) is reverse-transcribed by RT into double-stranded viral DNA (vDNA)[4,5]. RT initiates reverse transcription by using the positive-stranded vRNA as a template and the host Lys3 tRNA as a primer to synthesize a minus-strand DNA[5]. As RT synthesizes the vDNA the RNA template is degraded except for a purine-rich sequence known as the polypurine tract, which RT uses a primer for synthesis of the plus-strand DNA while using the completed minus-strand DNA as a template[5]. After reverse transcription is completed a multimer of IN binds either end of the linear vDNA and inserts it into the host chromosome by IN as a provirus[6].

A number of substitutions in IN (class II IN substitutions) or deletion of IN can interfere with virion morphogenesis and lead to morphologically aberrant viral particles with the vRNP mislocalized outside the capsid within the particles, referred to as eccentric viral particles[7-14]. Treating producer cells with a class of IN-targeting compounds, allosteric integrase inhibitors (ALLINIs), leads to similar morphological defects

in virions[8,9,15-22]. Intriguingly, eccentric virions generated by class II IN substitutions or ALLINI treatment are defective for reverse transcription in target cells[7-10,12,13,15,16,18,21,23-45] despite containing equivalent levels of RT and vRNA genome as wild type (WT) particles[8,46]. In addition, neither the condensation of the viral genome by NC[8,46] nor its priming[46] appear to be affected. Varying explanations have been offered for these observations. One possibility is that direct interaction between IN and RT is required for reverse transcription, a hypothesis that is bolstered by the finding that IN interacts with RT *in vitro*, and IN mutations that abolish this interaction also prevent reverse transcription in cells[39,45,47]. It is also possible that while vRNA is mislocalized outside the viral capsid in eccentric particles, RT is not, and physical separation of RT from vRNA underlies the defect in reverse transcription[48]. We and others have recently shown that premature loss of the viral genome and IN, as well as spatial separation of RT from vRNPs, may underlie the reverse transcription defect observed in eccentric viruses generated in the presence of ALLINIs or the class II IN R269A/K273A substitutions[48,49]. These findings support a model in which enclosure within the capsid lattice or IN binding to vRNA itself is necessary to protect viral components from the host environment upon entering the target cell. However, whether the premature loss of the viral genome and IN is a universal outcome of other class II IN substitutions is unknown.

In this work, we aimed to determine why eccentric viral particles produced by class II IN substitutions are blocked early in the viral life cycle. We found that all class II IN substitutions examined led to premature loss of the vRNA genome as well as IN itself in target cells. Additionally, there was a spatial separation of RT and CA from the vRNPs in target cells. Taken together, our findings suggest that a common mechanism underlies the loss of infectivity in class II IN mutant viruses and highlights the importance of the proper virion morphology for successful infection.

3.3 RESULTS

Class II IN mutant viruses are blocked early in the viral life cycle

Many class II IN substitutions are known to inhibit viral replication, and in particular prevent the completion of reverse transcription in target cells[7-10,12,13,15,16,18,23-45,50]. To test if the defect in reverse transcription is a common trait of class II IN substitutions, we first measured the titers of the class II IN mutant viruses previously generated (Chapter 2, Fig. 1). With the exception of E96A, nearly all of the IN substitutions reduced virus titers at least 100-fold compared to the WT (Figure 1A). We next measured the amount of vDNA in infected cells, and found a similar reduction in the amount of reverse-transcribed vDNA (Figure 1B). Although these results demonstrated a block in viral replication prior to integration, either at or before reverse transcription, we also sought to test the effects of the substitutions on the catalytic activity of IN. To assess whether the substitutions prevented IN from catalyzing the integration reaction in target cells, we trans-complemented a class I IN mutant virus defective for integration (NL4-3 IN D116N)[7,51] with each class II IN mutant protein using a previously described Vpr-IN fusion strategy[52,53]. In line with previous reports[25,26,33], the class II mutant IN molecules had variable levels of catalytic activity as assessed by their ability to rescue replication of the NL4-3 IN D116N virus. All Vpr-IN fusion proteins, except for the H12N mutant which likely decreased the stability of the Vpr-IN fusion protein, were expressed at similar levels in cells (Figure 1-Figure Supplement 1). We found that K14A, E96A, Y99A, K103A, V165A, R187A, K188E R199A, K236E, and R269A/R273A IN mutants trans-complemented a catalytically inactive IN at levels similar to the WT, whereas W108R, R228A, and V260E mutants were unable to do so (Figure 1C-D). The inability of W108R, R228A, and V260E mutants to trans-complement implies that they are impaired for integration, a result in line with previous observations[54,55]. The remainder of the IN mutants restored integration, albeit at significantly lower than WT levels (Figure 1C-D). These results suggest that the majority of the class II mutant INs retain structural integrity and at least partial catalytic activity in the presence of a complementing IN protein. Cumulatively, the data shows that class II IN substitutions

universally lead to the formation of non-infectious virions that are blocked at reverse transcription in target cells.

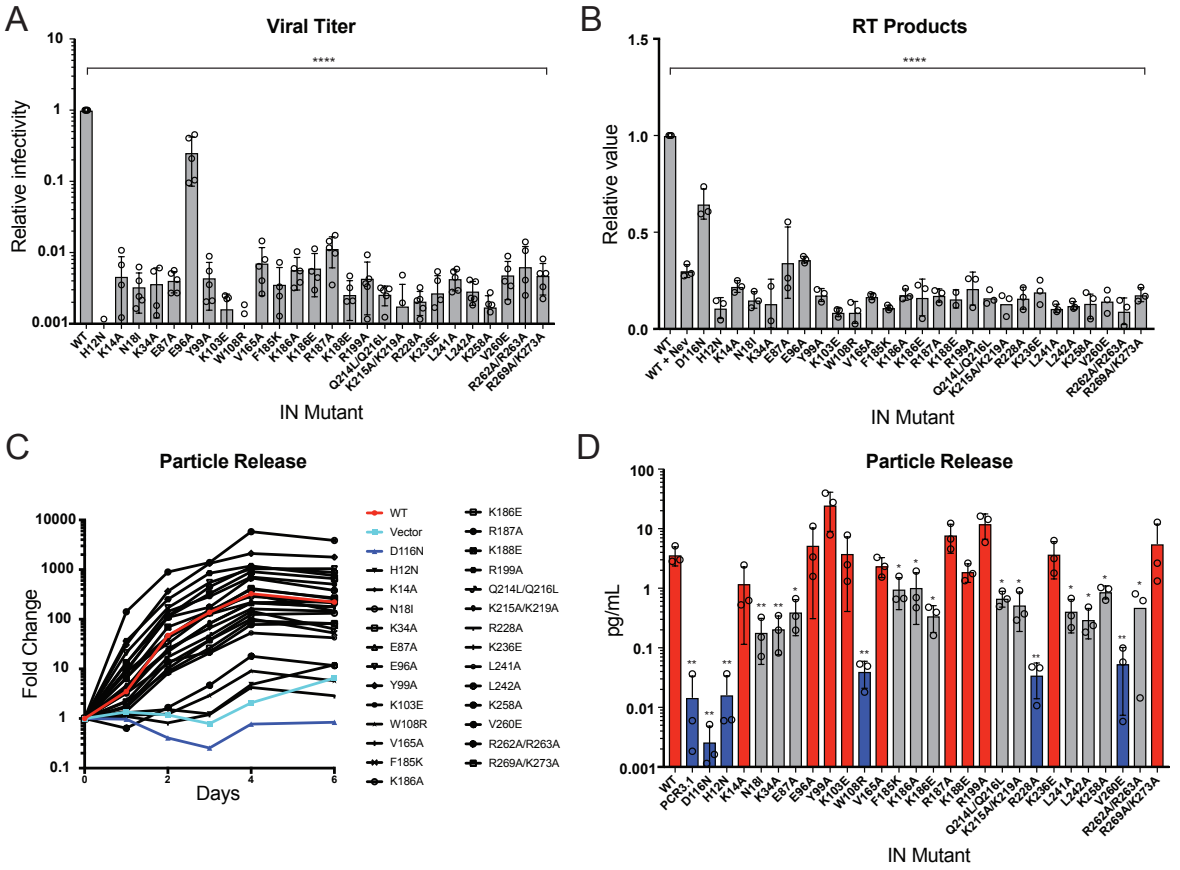

Figure 1: Class II IN mutant viruses are blocked early in the viral life cycle. (A) Infectious titers of WT or IN mutant HIV-1_{NL4-3} viruses in cell culture supernatants were determined on TZM-bl indicator cells. Titer values are expressed relative to WT (set to 1). Columns show average of five independent experiments (open circles) and error bars represent standard deviation (****P < 0.0001, by one-way ANOVA with Dunnett's multiple comparison test). (B) Relative quantity of reverse-transcribed HIV-1 DNA in MT-4 target cells infected with HIV-1_{NL4-3} at 6 hpi. Quantities of vDNA are expressed relative to WT (set to 1). Columns show average of three independent experiments (open circles) and error bars represent standard deviation (****P < 0.0001, by one-way ANOVA with Dunnett's multiple comparison test). (C) Representative growth curve of HIV-1_{NL4-3} IN_{D116N} viruses trans-complemented with class II mutant IN proteins in cell culture. Y-axis indicates fold increase in virion yield over day 0 as measured by RT activity in culture supernatants. HIV-1_{NL4-3} IN_{D116N} viruses that were trans-complemented with WT IN, class II mutant INs, IN_{D116N}, or an empty vector are denoted as red, black, dark blue and light blue lines respectively. Representative plot from one of three independent experiments. (D) Fold increase in virions in culture supernatants at 4 dpi, as measured by RT activity in culture supernatants. Trans-complementation of the HIV-1_{NL4-3} IN_{D116N} virus with mutant IN molecules restored particle release to levels comparable to WT IN (red), partially restored particle release (gray) or could not restore particle release (blue). Columns

show average of three independent experiments (open circles) and error bars represent standard deviation (*P < 0.05 and **P < 0.01, by paired t test between individual mutants and WT).

Viral RNA and IN is prematurely lost from cells infected with Class II IN mutant viruses

We have previously shown that vRNA and IN are prematurely lost from cells infected with the R269A/K273A class II IN mutant[48]. Given that eccentric vRNP localization is a common feature of class II IN mutant viruses (Chapter 2, Figure 6), we asked whether loss of vRNA in target cells is a common outcome for other class II IN mutant viruses. In contrast to the vRNA, the majority of mutant IN molecules appeared to remain associated with higher-order CA in virions (Chapter 2, Figure 7), and so we also wanted to test whether they would be protected from premature degradation in infected cells.

The fates of viral core components in target cells were tracked using a previously described biochemical assay[56]. For these experiments we utilized pgsA-745 cells (pgsA), which lack surface glycosaminoglycans, and likely as a result can be very efficiently infected by VSV-G-pseudotyped viruses in a synchronized fashion. PgsA cells were infected with WT or IN mutant viruses bearing substitutions that inhibited IN-vRNA interactions directly and may lead to mislocalization of IN in virions (i.e. K34A, R262A/R263A, R269A/K273A) or indirectly through aberrant IN multimerization and did not appear to grossly affect IN localization in virions (i.e. E87A, V165A) (Figure 2A). Two hours post infection, post-nuclear lysates were separated on linear sucrose gradients, and fractions collected from gradients were analyzed for viral proteins (CA, IN, RT) and vRNA by immunoblotting and Q-PCR-based assays, respectively.

As previously reported[48,56], in cells infected with WT viruses, IN, RT, vRNA and a fraction of CA comigrated in sucrose fractions 6-8, representing active RTCs (Figure 2B-E). Note that a large fraction of CA migrated in the top two soluble sucrose fractions representing CA that had dissociated from the core as a result of uncoating or CA that was packaged into virions but not incorporated into the capsid lattice[57,58]. Notably, in cells infected with class II IN mutant viruses, equivalent levels of CA (Figure 2B) and RT (Figure 2D) remained in the denser fractions, whereas IN (Figure 2C) and vRNA (Figure 2E) were

substantially reduced. Loss of vRNA and IN from dense fractions, without any corresponding increase in the top fractions containing soluble proteins and RNA, suggest their premature degradation and/or mislocalization in infected cells.

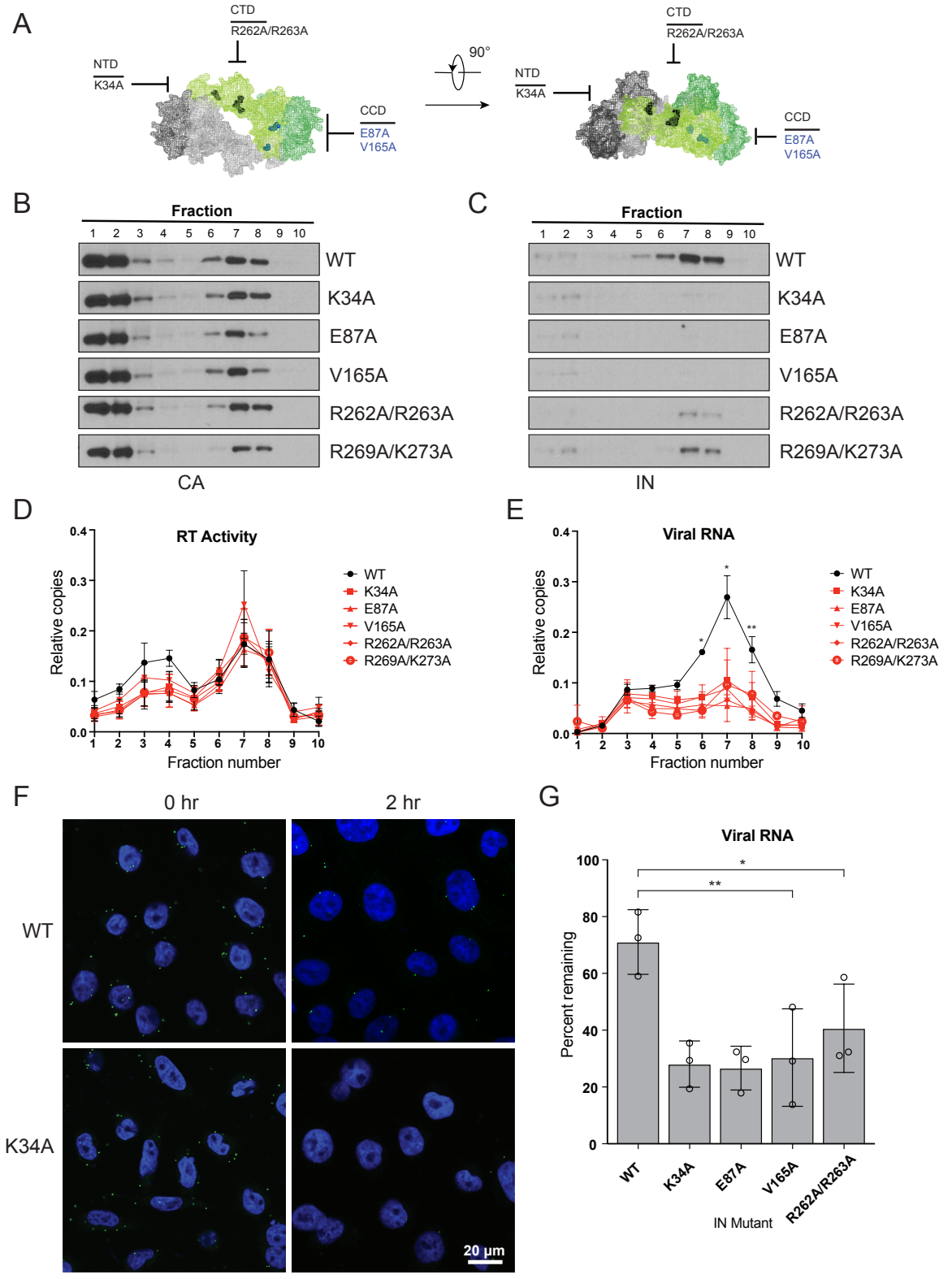

Figure 2. Premature loss of vRNA and IN from class II IN mutant viruses upon infection of target cells. (A) Locations of the class II IN substitutions K34A, E87A, V165A and R262A/R263A displayed on a single IN monomer within the context of the HIV-1 IN tetramer intasome structure (PDB 5U1C.)

Substitutions are color coded based on whether they may cause mislocalization of IN in virions (black) or not (blue.) (B-E) PgsA-745 cells were infected with WT or IN mutant HIV-1 virions and fates of viral core components were analyzed 2 hpi. Fractions were analyzed for the presence of CA (B) and IN (C) by immunoblotting and for RT activity (D) and vRNA (E) by Q-PCR. Immunoblots are representative of three independent experiments. Graphed data in (D) and (E) is the average of three independent experiments with error bars indicating standard deviation (*P < 0.05 and **P < 0.01, by repeated measures one-way ANOVA.) (F) Representative images of pgsA-745 cells infected with WT or IN mutant HIV-1_{NL4-3} viruses 0 and 2 hpi. Cells were stained for vRNA (green) and nuclei (blue) as detailed in Materials and Methods. (G) Quantification of vRNA remaining in cells infected with WT or IN mutant HIV-1_{NL4-3} viruses at 2 hpi. Values are the percent of vRNA remaining at 2 hpi compared to at 0 hpi. Columns show average of three independent experiments (open circles) and error bars represent standard deviation (*P < 0.05 and **P < 0.01, by one-way ANOVA with Dunnett's multiple comparison test.)

We next employed a complementary microcopy-based assay[59] in the context of full-length viruses to corroborate these findings. Advantages of this approach over biochemical fractionation experiments include the ability to track HIV-1 vRNA at the single-cell level with a high degree of specificity (Figure 2-Figure Supplement 1A), determine its subcellular localization, and to side-step possible post-processing artifacts associated with biochemical fractionation. Cells were synchronously infected with VSV-G pseudotyped HIV-1_{NL4-3} in the presence of nevirapine to prevent vRNA loss due to reverse transcription, and vRNA levels associated with cells immediately following synchronization (0 hour) and 2 hours post-infection were evaluated[59]. In WT-infected cells, vRNA was clearly visible immediately after infection (Figure 2F). Two hours post-infection, cell associated vRNA had fallen to 60-80% of starting levels (Figure 2F-G), likely as the result of some viruses failing to enter or perhaps being degraded after entry. However, a significant proportion of vRNA was still readily detectable. In contrast, in cells infected with the IN mutant viruses the reduction in vRNA was greater, and by 2 hours post-infection only 30-40% remained (Figure 2F-G, Figure 2 Supplement 1B). These results support the conclusion from the biochemical fractionation experiments that vRNA is prematurely lost from cells infected with class II IN mutant viruses.

Finally, we tested whether our findings held true in physiologically relevant human cells. MT-4 T cells were synchronously infected with WT or class II IN mutant VSV-G pseudotyped HIV-1_{NL4-3} in the presence of nevirapine. Cells were collected immediately after synchronization (0 hour), 2 and 6 hours post-infection, and the quantity of vRNA measured by Q-PCR. In line with the above findings, vRNA levels decreased at a faster rate with the class II IN mutants as compared to WT viruses, with half as much cell-

associated vRNA remaining at 2 and 6 hours post-infection for the class II IN mutants (Figure 3A). Treating cells with ammonium chloride to prevent fusion of the VSV-G pseudotyped viruses rescued vRNA loss, and vRNA from WT and mutant viruses were retained at equal levels, indicating that the loss of vRNA is dependent on entry into the target cell (Figure 3B). These findings agree with the previous experiments and demonstrate that class II IN substitutions lead to the premature loss of vRNA genome also in human T cells.

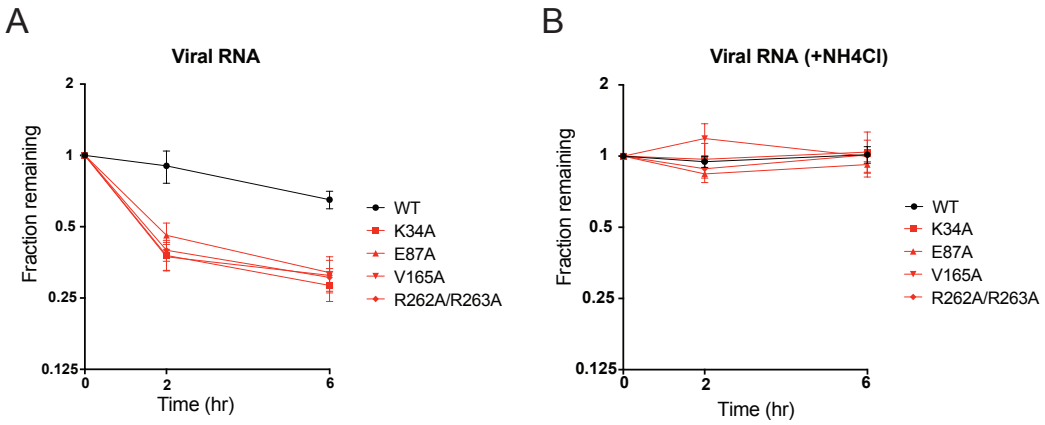

Figure 3. Loss of vRNA from class II IN mutant viruses upon infection of target cells is dependent on viral entry. (A) Fraction of viral RNA remaining after 2 and 6 hpi compared to the quantity measured at 0 hpi. MT-4 cells were synchronously infected with VSV-G pseudotyped HIV-1_{NL4-3} viruses and at each timepoint samples of infected cultures were taken for analysis. Viral RNA levels in samples were measured by Q-PCR and normalized to the levels of GAPDH mRNA. Data points are the average of five independent experiments with error bars indicating standard error of the mean. (B) Fraction of viral RNA remaining after 2 and 6 hpi compared to the quantity measured at 0 hpi as above. MT-4 cells were synchronously infected with VSV-G pseudotyped HIV-1_{NL4-3} viruses and incubated in the presence of 50 mM ammonium chloride for 6 hrs. Data points are the average of three independent experiments with error bars indicating standard error of the mean.

3.4 DISCUSSION

Our studies provide the mechanistic basis for how diverse class II IN mutations result in a common block in viral replication. We propose premature degradation of the exposed vRNA and IN, as well as separation of RT from the vRNA, underlies the reverse transcription defect seen in class II IN mutant viruses. In addition to class II IN mutations, it is likely that our findings also apply to eccentric viruses produced by ALLINI treatment, and help elucidate how these compounds exert their antiviral effects.

Several studies to date have found that when class II IN mutant viruses or ALLINI-treated viruses enter target cells, vRNA and IN itself is lost from the cells using both biochemical[48] and microscopy-based assays[49]. However, this is the first study to our knowledge to characterize the replication defects of a large and diverse panel of class II IN mutant viruses side-by-side. Our results and those of others indicate that the premature loss of vRNA and IN in target cells is a common result of eccentric virion morphology. Interestingly, mutations in CA that destabilize the capsid lattice *in vitro* also block reverse transcription in target cells[60-63], and treating producer cells with a CA-targeting compound leads to the generation of eccentric viral particles defective for reverse transcription[64], much like viral particles produced in the presence of ALLINIs. Finally, destabilizing CA mutations lead to the premature loss of vRNA and IN from target cells[65], just like class II IN mutations. Taken together, these findings argue that proper encapsidation within the viral core is necessary to protect vRNA and viral replicative proteins from the host cell environment, and when unprotected by the viral capsid, vRNA and IN are either passively or actively degraded.

A mechanism responsible for the loss of vRNA and IN in target cells has yet to be defined. It is possible that the high AU-content of HIV-1 vRNA makes it inherently unstable[66-68], in a manner similar to that of cellular mRNAs that encode cytokines and growth factors[69]. It is also possible that when ectopically expressed alone in cells, IN undergoes proteasomal degradation[70-74], and knockdown of a cellular component of the ubiquitin-conjugation system, E3 RING ligase TRIM33, enhances HIV-1 infection and replication in cells[71]. However, another study found that during infection with eccentric viral particles, the loss of vRNA and IN was proteasome-independent[48]. Future studies are warranted to determine the precise mechanism of vRNA and IN degradation upon their premature exposure.

In addition to preventing premature degradation of vRNA and IN, the CA lattice may also play an important role in shielding viral core components from host immune recognition. Mutating the HIV-1 CA to prevent its interaction with cellular factors leads to activation of innate immune responses and cytokine production in infected dendritic cells[75] and activation of a type 1 interferon response in infected monocyte-

derived macrophages[76], suggesting that the CA lattice is important for recruiting cellular proteins to cloak viral components from detection. While it is important to note that infected dendritic cells appear to detect reverse-transcribed viral DNA through the cytosolic DNA sensor cGAS[75], innate cytokine expression is also elicited in peripheral blood mononuclear cells transfected with purified HIV-1 RNA[77], demonstrating that HIV-1 RNA does have the potential to trigger immune activation.

In conclusion, we have identified the premature loss of the vRNA genome and IN, as well as spatial separation of the vRNA and RT, as probable underlying causes for block in virus replication seen in class II IN mutant viruses. Our findings elucidate why proper virion morphology is important for viral infectivity, and support the concept of targeting virion maturation as a therapeutic strategy.

3.5 MATERIALS AND METHODS

Key Reagents

Key Reagents				
Reagent type (species)	**Designation**	**Source or reference**	**Identifiers**	**Additional information**
Gene (human immunodeficiency virus type 1)	Integrase (IN)	NCBI (NC_001802.1)	Gene ID: 155348	
Strain, strain background (*Escherichia coli*)	DH10B	Thermo Fisher Scientific	EC0113	Competent cells
Strain, strain background (*Escherichia coli*)	BL21	Thermo Fisher Scientific	C600003	Competent cells
Cell line (*Homo sapiens*)	HEK293T	ATCC	CRL-11268	
Cell line (*Homo sapiens*)	TZM-bl	NIH AIDS Reagent Program	8129	
Cell line (*Cricetulus griseus*)	pgsA-745	ATCC	CRL-2242	Xylosyltransferase I deficient
Cell line (*Homo sapiens*)	MT-4	NIH AIDS Reagent Program	120	
Antibody	Anti-HIV-1 integrase-4 (mouse monoclonal)	Bouyac-Bertoia et al., 2001		WB (1:4000)
Antibody	Anti-HIV-1 p24 antibody (mouse monoclonal)	NIH AIDS Reagent Program	183-H12-5C	WB (1:100)
Commercial assay, kit	QuikChange Site-Directed Mutagenesis Kit	Agilent Technologies	Cat# 200519	
Commercial assay, kit	DNeasy Blood and Tissue kit	Qiagen	Cat# 69506	
Commercial assay, kit	RNAScope F luorescent Multiplex Detection Reagents	Advanced Cell Diagnostics	Ref# 320851	
Chemical compound, drug	Nevirapine	NIH AIDS Reagents		

Plasmids

The pNLGP plasmid consisting of the HIV-1_{NL4-3} -derived Gag-Pol sequence inserted into the pCR/V1 plasmid backbone[78] and the CCGW vector genome plasmid carrying a GFP reporter under the control of the CMV promoter[79,80] were previously described. The pLR2P-vprIN plasmid expressing a Vpr-IN fusion protein has also been previously described[53]. Mutations in the IN coding sequence were introduced into both the pNLGP plasmid and the HIV-1_{NL4-3} full-length proviral plasmid (pNL4-3) by overlap extension PCR. Briefly, forward and reverse primers containing IN mutations in the *pol* reading frame were used in PCR reactions with antisense and sense outer primers containing unique restriction endonuclease sites (AgeI-sense, NotI-antisense for NLGP and AgeI-sense, EcoRI-antisense for pNL4-3), respectively. The resulting fragments containing the desired mutations were mixed at 1:1 ratio and overlapped subsequently using the sense and antisense primer pairs. The resulting fragments were digested with the corresponding restriction endonucleases and cloned into pNLGP and pNL4-3 plasmids. IN mutations were introduced into the pLR2P-vprIN plasmid using the QuickChange Site-Directed Mutagenesis kit (Agilent Technologies). Presence of the desired mutations and absence of unwanted secondary changes were verified by Sanger sequencing.

Cells and viruses

All cell lines were originally obtained from American Type Culture Collection and NIH AIDS Reagents where STR profiling was performed. MT-4 cells were additionally subjected to STR profiling at Washington University School of Medicine Genome Engineering and iPSC center. All cell lines are regularly checked for mycoplasma contamination using the MycoAlert mycoplasma detection kit (Lonza) and verified to be free of contamination during the course of these studies. HEK293T cells (ATCC CRL-11268) and HeLa-derived TZM-bl cells (NIH AIDS Reagent Program) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. MT-4 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. CHO K1-derived pgsA-745 cells (CRL-2242, ATCC) that lack a functional xylosyltransferase enzyme and as a result do not produce

glycosaminoglycans were maintained in Dulbecco's modified Eagle's / F12 (1:1) media supplemented with 10% fetal bovine serum and 1 mM L-glutamine. Single-cycle GFP reporter viruses pseudotyped with vesicular stomatitis virus G protein (VSV-G) were produced by transfection of HEK293T cells with pNLGP-derived plasmids, the CCGW vector genome carrying GFP, and VSV-G expression plasmid at a ratio of 5:5:1, respectively, using polyethyleneimine (PolySciences, Warrington, PA). Full-length viruses pseudotyped with VSV-G were produced by transfecting HEK293T cells with the pNL4-3-derived plasmids and VSV-G plasmid at a ratio of 4:1 (pNL4-3:VSV-G).

Immunoblotting

Viral and cell lysates were resuspended in sodium dodecyl sulfate (SDS) sample buffer and separated by electrophoresis on Bolt 4-12% Bis-Tris Plus gels (Life Technologies), blotted onto nitrocellulose membranes and probed overnight at 4°C with the following antibodies in Odyssey Blocking Buffer (LI-COR): mouse monoclonal anti-HIV p24 antibody (183-H12-5C, NIH AIDS reagents) and mouse monoclonal anti-HIV integrase antibody[81]. For analysis of the fates of core components in infected cells, antibody incubations were done using 5% non-fat dry milk. Membranes were probed with HRP-conjugated secondary antibodies and developed using SuperSignal™ West Femto reagent (Thermo-Fisher).

Analysis of reverse transcription products in infected cells

MT-4 cells were grown in 24-well plates and infected with VSV-G pseudotyped pNL4-3 viruses (either WT or class II IN mutant) at a multiplicity of infection (MOI) of 2 in the presence of polybrene. Six hr post-infection cells were collected, pelleted by brief centrifugation, and resuspended in PBS. DNA was extracted from cells using the DNeasy Blood and Tissue Kit (Qiagen) as per kit protocol. Quantity of HIV-1 vDNA was measured by Q-PCR using primers specific for early reverse-transcripts.

Vpr-IN transcomplementation experiments

A class I IN mutant virus (HIV-1_{NL4-3} IN_{D116N}) was trans-complemented with class II mutant IN proteins as described previously[53]. Briefly, HEK293T cells grown in 24-well plates were co-transfected with a derivative of the full-length HIV-1_{NL4-3} proviral plasmid bearing a class I IN substitution (pNL4-3_{D116N}), VSV-G, and derivatives of the pLR2P-vprIN plasmid bearing class II IN mutations at a ratio of 6:1:3. Two days post-transfection cell-free virions were collected from cell culture supernatants. Integration capability of the trans-complemented class II IN mutants was tested by infecting MT-4 cells and measuring the yield of progeny virions in cell culture supernatants over a 6-day period as described previously[53]. In brief, MT-4 cells were incubated with virus inoculum in 96 V-bottom well plates for 4 hr at 37°C after which the virus inoculum was washed away and replaced with fresh media. Immediately following removal of the virus inoculum and during the six subsequent days the quantity of virions present in the culture supernatant was quantified by measuring RT activity using a Q-PCR-based assay[82].

Biochemical analysis of virion core components in infected cells

Biochemical analysis of retroviral cores in infected cells was performed as described previously[56]. Briefly, pgsA-745 cells were infected with VSV-G pseudotyped single cycle GFP-reporter viruses or its derivatives synchronously at 4°C. Following the removal of virus inoculum and extensive washes with PBS, cells were incubated at 37°C for 2 hr. To prevent loss of vRNA due to reverse-transcription, cells were infected in the presence of 25 μM nevirapine. Post-nuclear supernatants were separated by ultracentrifugation on 10-50% linear sucrose gradients using a Beckman SW55-Ti rotor at 30,000 rpm for 1 hr at 4°C. Ten 500 μl fractions from the top of the gradient were collected, and CA, IN, and vRNA in each fraction were analyzed by either immunoblotting or Q-PCR[56]. A SYBR-Green-based Q-PCR assay[82] was used to determine RT activity in the collected sucrose fractions.

Visualization of vRNA in infected cells

Viral RNA was visualized in infected cells according to the published multiplex immunofluorescent cell-based detection of DNA, RNA and Protein (MICDDRP) protocol[59]. VSV-G pseudotyped HIV-1_{NL4-3} virus stocks were prepared as described above and concentrated 40X using a lentivirus precipitation solution (ALSTEM). PgsA-745 cells were plated on 1.5 mm collagen-treated coverslips (GG-12-1.5-Collagen, Neuvitro) placed in 24-well plates one day prior to infection. Synchronized infections were performed by incubating pre-chilled virus inoculum on the cells for 30 min at 4°C. Cells were infected with WT virus at a MOI of 0.5, or with an equivalent number (normalized by RNA copy number) of IN mutant viral particles. After removal of the virus inoculum cells were washed with PBS and either immediately fixed with 4% paraformaldehyde, or incubated at 37°C for 2 hr before fixing. To prevent loss of vRNA due to reverse-transcription, cells were infected and incubated in the presence of 25 μM nevirapine. Following fixation, cells were dehydrated with ethanol and stored at -20°C. Prior to probing for vRNA, cells were rehydrated, incubated in 0.1% Tween in PBS for 10 min, and mounted on slides. Probing was performed using RNAScope probes and reagents (Advanced Cell Diagnostics). Briefly, coverslips were treated with protease solution for 15 min in a humidified HybEZ oven (Advanced Cell Diagnostics) at 40 °C. The coverslips were then washed with PBS and pre-designed anti-sense probes[59] specific for HIV-1 vRNA were applied and allowed to hybridize with the samples in a humidified HybEZ oven at 40 °C for 2 hr. The probes were visualized by hybridizing with preamplifiers, amplifiers, and finally, a fluorescent label. First, pre-amplifier 1 (Amp 1-FL) was hybridized to its cognate probe for 30 min in a humidified HybEZ oven at 40 °C. Samples were then subsequently incubated with Amp 2-FL, Amp 3-FL, and Amp 4A-FL for 15 min, 30 min, and 15 min respectively. Between adding amplifiers, the coverslips were washed with a proprietary wash buffer. Nuclei were stained with DAPI diluted in PBS at room temperature for 5 min. Finally, coverslips were washed in PBST followed by PBS and then mounted on slides using Prolong Gold Antifade.

Microscopy and image quantification

Images were taken using a Zeiss LSM 880 Airyscan confocal microscope equipped with a ×63/1.4 oil-immersion objective using the Airyscan super-resolution mode. 10 images were taken for each sample using the ×63 objective. Numbers of nuclei and vRNA punctae in images were counted using Volocity software (Quorum Technologies). The number of vRNA punctae per 100 nuclei were recorded at 0 hr post-infection (hpi) and 2 hpi for each virus, and the number at 2 hpi compared to the number at 0 hpi.

Analysis of the fate of vRNA genome in MT4 cells

MT-4 cells were infected with VSV-G pseudotyped HIV-1 NL4-3 WT or equivalent number of mutant viruses (normalized by RT activity) synchronously at 4°C. After removal of virus inoculum and extensive washes with PBS, cells were incubated at 37°C for 6 hr in the presence of 25 μM nevirapine. Immediately after synchronization (0 h) and at 2 and 6 hr post-infection samples were taken from the infected cultures and RNA was isolated using TRIzol Reagent. The amount of viral genomic RNA was measured by Q-RT-PCR.

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3.7 SUPPLEMENTAL FIGURES

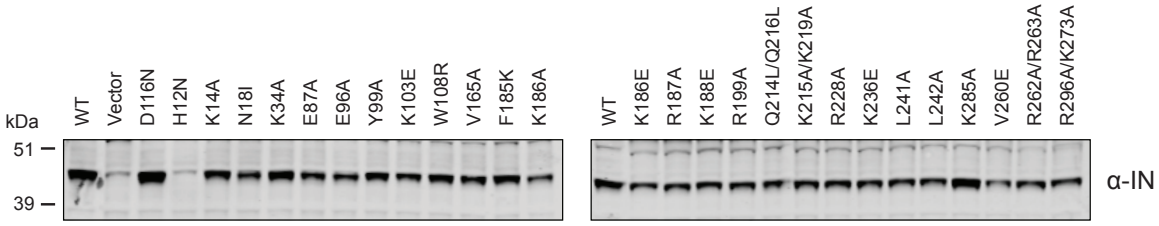

Figure 1-Figure Supplement 1. Expression of Vpr-IN fusion constructs. Representative immunoblot analysis of Vpr-IN fusion constructs in cell lysates. HEK293T cells were co-transfected with the HIV-1_{NL4-3} IN_{D116N} proviral plasmid along with Vpr-IN expression plasmids encoding for the indicated IN substitutions or an empty vector control. Expression of Vpr-IN constructs in cell lysates was detected using an anti-IN antibody.

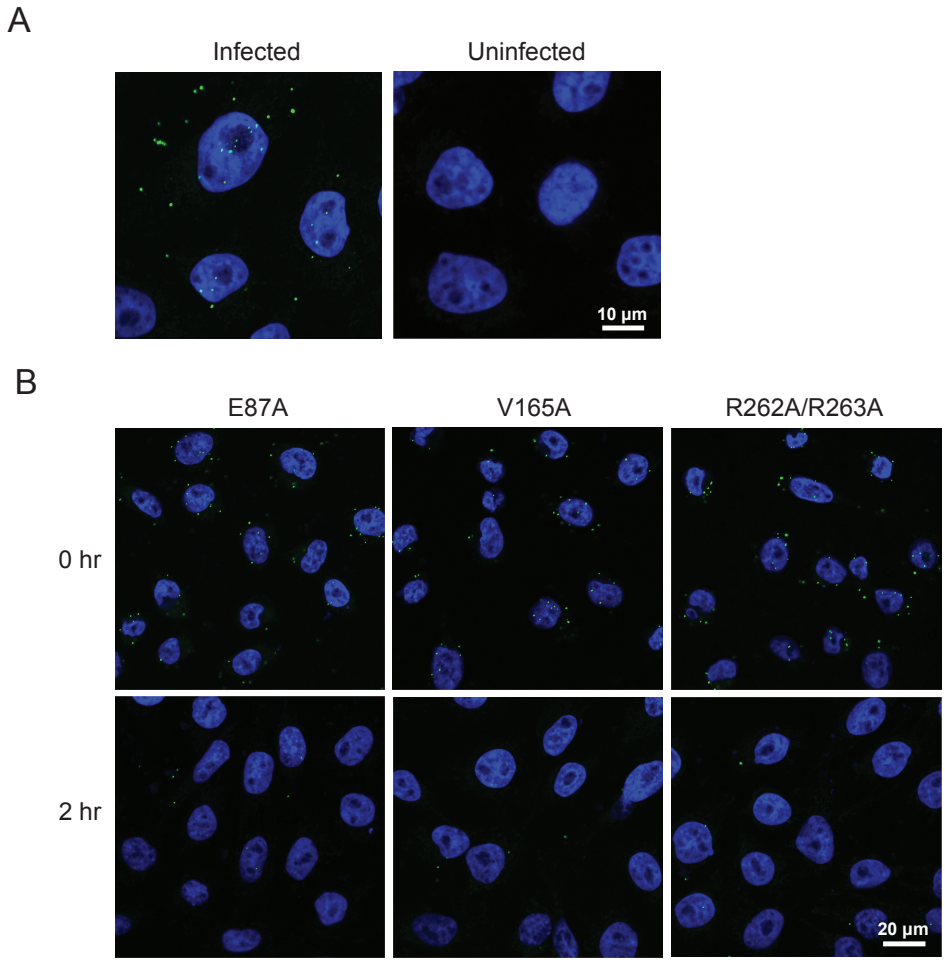

Figure 2-Figure Supplement 1. Premature loss of vRNA and IN from class II IN mutant viruses upon infection of target cells. (A) Representative images of uninfected pgsA-745 cells and cells infected with WT HIV-1_{NL4-3} viruses at 0 hpi. Cells were fixed and stained for vRNA (green) and nuclei (blue). (B) Representative images of pgsA745 cells infected with IN mutant HIV-1_{NL4-3} viruses 0 and 2 hpi. Cells were fixed and stained for vRNA (green) and nuclei (blue).

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Chapter 4:

Summary and Future Investigations

4.1 SUMMARY

This dissertation examines the mechanisms by which the HIV-1 integrase enzyme (IN) contributes to virion morphogenesis and viral replication independent of its function in integration. The findings presented in Chapter 2 demonstrate that IN ensures proper packaging of the HIV-1 RNA genome (vRNA) within the viral capsid via its binding to vRNA, and that IN-RNA binding requires proper IN multimerization. The data presented in Chapter 3 provides evidence that improperly formed viral particles generated by IN mutations are unable to replicate in target cells due to the premature loss of the exposed vRNA and IN. Collectively, these studies illuminate the vital role of the of IN in virion morphogenesis and the viral life cycle independent of its canonical role in integration.

IN-RNA interaction accounts for the role of IN in virion morphogenesis

Early mutagenesis studies have found that multiple substitutions in IN (class II mutations) lead to the generation of eccentric viral particles with the viral ribonucleoprotein complex (vRNP) consisting of the vRNA and nucleocapsid protein (NC) mislocalized outside of the capsid[1-6]. These obsvertaions provided the first hints that IN played a key role in virion maturation. However, a mechanistic explanation for why IN is crucial for proper particle formation remained elusive until the recent discovery that IN binds vRNA in virions at its CTD, and that preventing IN-RNA binding leads to the generation of eccentric viral particles[7]. This finding argued that IN mediates the correct placement of vRNA within the capsid through is interaction with vRNA; however, it remained unanswered how various mutations along the length of IN (class II mutations) cause the same morphological defects in virions despite being distally located from the RNA-binding site in the IN CTD. Our findings in Chapter 2 demonstrate that all class II IN mutations examined prevent IN-RNA binding in virions, and do so by one of three mechanisms; i.) by reducing the levels of IN in virions and precluding the formation of IN-RNA complexes; ii.) by interfering with proper IN multimerization and thereby indirectly preventing IN binding RNA; iii.) by directly preventing IN-RNA binding without affecting IN levels or multimerization. We further show that IN binds RNA as a tetramer, and that tetramerization is likely required to the binding and condensation of

vRNA during virion maturation. Our results cement the conclusion that IN-RNA binding accounts for the role of IN in virion morphogenesis, and provide mechanistic insight on how IN binds RNA.

vRNA and IN from class II IN mutant viruses are prematurely lost from target cells

Eccentric viral particles are non-infectious, and are blocked at or prior to the reverse transcription step in target cells[1,3,5-35]. Our findings in Chapter 3 reveal that in cells infected with class II IN mutant viruses vRNA and IN are prematurely lost. In addition, there was a spatial separation of reverse transcriptase (RT) from vRNA. Either the loss of the vRNA genome or its separation from RT would prevent successful reverse transcription and further viral replication. In addition, the loss of IN would prevent the downstream integration of the reverse-transcribed vDNA into the host DNA. Finally, we show that the loss of vRNA occurs after viral entry. Our results provide an explanation for why eccentric viral particles are defective for reverse transcription and unable to complete the HIV-1 life cycle, and suggest that the viral capsid is necessary to protect vRNA and IN after entry into the target cell.

4.2 FUTURE INVESTIGATIONS

Temporal assessment of IN-RNA interaction during virion morphogenesis

The original discovery that HIV-1 IN binds viral genomic RNA in virions[7] together with the evidence presented in Chapter 2 support the conclusion that IN contributes to proper virion maturation through its interaction with RNA. However, the temporal events during the maturation process and exactly when IN begins to interact with RNA remains unclear. As reviewed in Chapter 1, IN is synthesized as a domain of the Gag-Pol precursor polyprotein, and does not exist as an independent protein until it is cleaved by PR during virion maturation. It remains unanswered whether the IN domain can bind RNA in the context of the Gag-Pol precursor in cells or immature virions, or whether it must first be cleaved by PR. To determine if IN binds RNA in infected producer cells or immature virions, future studies will utilize a full length NL4-3 proviral clone lacking PR and with Factor Xa cleavage sites inserted in the Gag-Pol polyprotein around the IN domain. In the absence of PR, the Gag and Gag-Pol polyproteins will not be

cleaved and virions will remain trapped in an immature state after budding. In order to liberate the IN domain from the rest of Gag-Pol, cell and virion lysates will be treated with Factor Xa. The then separated IN domain can then be immunoprecipitated, along with any bound RNA. This approach will be combined with CLIP[36] to immunoprecipiate IN and determine if the IN domain of Gag-Pol binds RNA in the producer cells or immature virions.

Previous studies[4] and the data presented in Chapter 2 (Figure 6) demonstrate that deleting IN entirely results in the formation of both eccentric and immature viral particles. As the immature particles produced by the IN-deleted virus appear morphologically similar to immature WT viral particles before maturation, I predict that IN is not necessary for correct morphogenesis until the maturation step, and that IN does not interact with RNA until it is cleaved from the Gag-Pol precursor polyprotein. If this is the case, no vRNA should coimmunoprecipitate with the IN domain from producer cells or immature virions. However, if the proposed CLIP experiments do demonstrate that the IN domain of Gag-Pol binds vRNA in producer cells or immature virions, next-generation RNA sequencing as detailed in the CLIP protocol[36] will be used to determine the regions of vRNA bound by IN and identify any secondary structures or sequence motifs.

Localization of IN in eccentric viral particles

The results of the experiments presented in Chapter 3 (Figure 2) show that in target cells infected with class II IN mutant viruses IN itself is lost along with viral RNA, while data from Chapter 2 (Figure 7) shows that in a few class II IN mutant viruses IN becomes separated from the rest of the viral core when viral particle components are separated over linear sucrose gradients, suggesting that IN may also be mislocalized in the viral particles. However, in the majority of class II IN viruses IN migrated over the sucrose gradients similarly to the WT, and so it remains unclear if IN is located within or outside the capsid in mutant viral particles.

In contrast to vRNA which can be clearly visualized in virions using transmission electron microscopy (TEM), IN is not easily visualized by TEM and determining its exact localization within viral

particles is challenging. Attempts to label and visualize IN in viral particles using immuno electron microscopy (immune EM) were not successful, and so we next attempted to explain why IN migrates the way it does over the sucrose gradients. Neither destabilizing the viral capsid (Chapter 2, Figure 7 -Figure Supplement 1A) nor generation of virions devoid of RNA (Chapter 2, Figure 7 -Figure Supplement 1B) affected how IN migrated over linear sucrose gradients, indicating that the migration of IN over the gradients is independent of its being enclosed in an intact capsid or its being bound to RNA. However, these results do not offer any further insight into where IN is located in viral particles. While tagging IN often adversely affects viral fitness, one paper reports successfully labelling IN with and HA tag[37], allowing for IN to be labeled in infected cells using immune EM. Future investigation of where IN is located in viral particles could employ a similar strategy.

Conservation of IN-RNA interaction across different retroviruses

Although all retroviruses initially produce immature viral particles that undergo a maturation process shortly after budding from the infected cell[38], there is considerable variation in the morphologies of the mature capsids[39,40]. Whether IN plays a role in virion maturation in other retroviruses has not been investigated. Intriguingly, mutations in the C-terminal domain of the murine leukemia virus (MLV) IN prevent reverse transcription in target cells similar to class II mutations in HIV-1[41,42]. More study is needed to determine if IN contributes to proper virion maturation in other retroviruses, and if IN does so by binding to RNA. Future research will use the previously mentioned CLIP protocol[36] to determine if IN binds vRNA in mature virions of other retroviruses, and will test if inhibiting IN-RNA interactions leads to the generation of morphologically aberrant viral particles.

Defining mechanisms responsible for the loss of vRNA and IN in target cells

In Chapter 3 we demonstrate that both vRNA and IN are prematurely lost from target cells infected with class II IN mutant viruses. In addition, vRNA becomes spatially separated form RT. Either the loss of vRNA or its separation from RT would be sufficient to prevent reverse transcription and further viral

replication. TEM experiments clearly show that vRNA is mislocalized outsde the capsid in class II IN mutant viruses (Chapter 2, Figure 6), and when lysates from infected cells are separated over sucrose gradients RT comigrates with the viral capsid (CA) protein (Chapter 3, Figure 2), suggesting that RT is retained inside the viral capsid. However, we do not further define mechanisms responsible for the loss vRNA and IN.

We have previously shown that proteasomal inhibition does not rescue vRNA and IN in target cells infected with eccentric particles generated by treatment with an allosteric integrase inhibitor (ALLINI) or the IN R269A/K273A mutation[43], and I suspect that the loss of vRNA and IN is proteasome-independent in other class II IN mutant viruses as well. However, the loss of vRNA can be rescued by preventing viral entry (Chapter 3, Figure 3B), indicating that a cellular mechanism may be involved. A possible candidate is the autophagy pathway, which has been implicated in the degradation of other HIV-1 proteins[44]. Future investigation may test if the loss of vRNA and IN is dependent on autophagy by infecting cells with class II IN mutant viruses in the presence of an autophagy inhibitors such as chloroquine or anti-proteases.

Determining the fate of CA mutant viral particles

Mutations in IN or treatment with ALLINI's disrupt virion morphogenesis and lead to the creation of eccentric viral particles that are blocked at reverse transcription in target cells. However, treating producer cells with compounds that target the viral CA protein itself can also lead to the generation of morphologically eccentric viral particles[45]. In addition, mutations in CA that destabilize the capsid lattice *in vitro* also block viral replication at reverse transcription[46-49]. The similar defects in both IN and CA mutant viruses suggest a shared underlying causal mechanism, and it is reasonable to imagine that either the mislocalization of the vRNA outside the capsid or the instability of the capsid after viral entry into the target cell may leave the vRNA exposed and vulnerable to loss or degradation. Ongoing experiments with CA mutant viruses have found that mutations that destabilize the capsid lattice also lead to the premature loss of vRNA and IN in target cells, similar to class II IN mutants[50]. Further investigation could focus on

identifying the mechanisms responsible for the loss of vRNA and IN, and test if they are the same mechanisms behind the loss of vRNA and IN in cells infected with class II IN mutant viruses.

Immune consequences of eccentric morphology

The capsid lattice is proposed not only to protect the vRNA and replicative enzymes from the host environment, but also to conceal the virus from immune recognition[51]. Cells are equipped with multiple cytosolic pattern recognition receptors (PRRs) capable of detecting viral RNA and DNA, and several PRRs including RIG-I[52] and cGAS[53] have been implemented in the sensing of HIV-1 nucleic acids. Transfecting purified HIV-1 RNA into peripheral blood mononuclear cells activates the NF-κB, p38, and IRF signaling pathways and elicits innate immune cytokine expression[52], demonstrating that HIV-1 RNA does have the potential to trigger a cell-intrinsic innate immune response. Whether the mislocalization of the vRNA outside of the capsid lattice in eccentric viral particles allows for the vRNA to be sensed and initiates immune activation in the host cell is unclear. It is conceivable that infection with eccentric viral particles may result in immune activation. Conversely, it is also possible that exposed HIV-1 vRNA is lost or degraded too quickly to adequately trigger an immune response. Much work remains to be done to determine whether infection with eccentric HIV-1 viral particles has any immune consequences. Considering that disrupting virion morphogenesis and generating non-infectious, morphologically eccentric viral particles may be a valuable therapeutic strategy, it will be important to know how the host immune system might respond to the presence of significant numbers of eccentric viral particles.

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